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CANCER RESEARCH

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Conditioned and Autonomous Neoplasms: *A Review**†

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Most current theories regard the basic alteration in cancer as residing not in the host but in the neoplastic cell, be this alteration a somatic mutation or an abnormal differentiation or affliction of cells with viruses or self-perpetuating particles. In this review tumors will be surveyed which find their origin in alteration of the host. Such tumors grow with little or no apparent restraint in altered hosts but not in normal hosts. In such neoplasms, called dependent or conditioned, some cells ultimately become altered and give rise to autonomous[1] growth. Evidence will be presented to indicate that autonomy is a quantitative characteristic. Quantitation of autonomy and dependence is possible in pituitary tumors secreting thyroid stimulating hormone (TSH), which arise in the absence of thyroid hormone (TH) and are maintained by suppression of TH production (41).

A complete survey of the pertinent literature is beyond the scope of this review and the capacity of the reviewer. In releasing this review I am motivated by the philosophy that to seek the perfect is to lose the good. A wealth of pertinent information is gathered in the books of Loeb (84), Allen *et al.* (2), Burrows (20), Willis (129), Twombly and Pack (126), and Lipschutz (83) and in many reviews including those of Gardner (48), Rous and Kidd (107), Lipschutz (84), and Rusch and Kline (111). The aim of this review is to contrast conditioned or dependent with autonomous neoplasms, which are often but stages of the same growth yet so basically different as to require different approaches for control: one to restore the disturbed equilibrium in the host, the other to destroy the altered cell.

In the adult organism a harmonious relationship (milieu interne of Claude Bernard) is established among the various cells by a mechanism termed homeostasis (Walter Cannon, 21). The growth of every cell in the body is undoubtedly regulated, though knowledge of this regulation is scanty; how the number of various types of cells is maintained at a desired level in a multicellular organism is a problem common to physiology and cancer research.

It has long been postulated that every cell in the body gives rise to substances which affect adjacent cells. The term "hormone" relates in a narrow sense to substances released by cells and acting at distant sites. In a broader sense it applies also to substances released at certain points to affect adjacent cells, e.g., neurohormones. Ribbert (106) was the first to postulate that a malignant change can originate in loss of growth restraint by adjacent tissue, enabling cells to proliferate and give rise to tumors. In this limited sense Ribbert's concept of growth restraint has been rejected, the favored arguments being that normal cells do not behave as cancer cells at abnormal sites and that cancer cells grafted into normal hosts will proliferate with no restraint (Willis, 130). In a broader sense Ribbert's view does, however, apply to many neoplasms of endocrine organs, and lack of restraint can be a major force at the early phase of evolution of many cancers. The validity of this view cannot be tested by a study of transplanted "autono-

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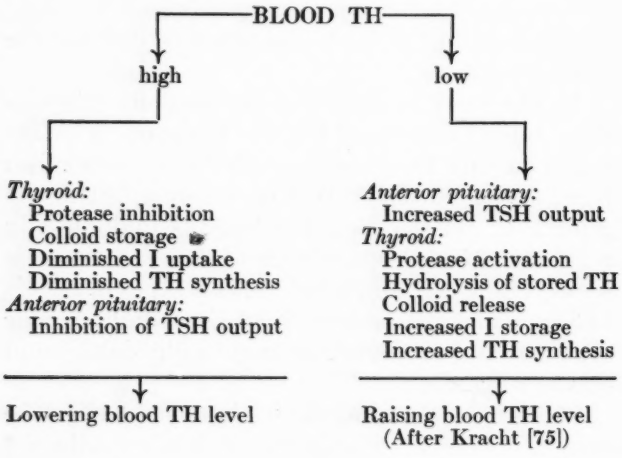
[1] The suggestion of Dr. Joseph Aub to drop the "ugly word of autonomy" from our nomenclature has its merit. The term is used as a hypothetical extreme in contrast to dependency, with the understanding that while one can prove dependency, one is never certain of autonomy.

mous" tumors which are the final products in the evolution of carcinogenesis reversible at earlier phases.

Cells of the epidermis are doubtless held in check by subjacent cells. Erythrocyte levels are to some extent controlled at about 5,000,000 per c. mm. by a given oxygen tension; leukocyte levels are well maintained at about 7,000 by an unknown regulator; cells secreting hormones are controlled by other hormones and by the metabolites or secretions of their "target" organs. For example, cells of the parathyroid are influenced by the plasma level of Ca and P, which are in equilibrium with tissue levels of Ca and P. The number of cells of "target" organs is controlled by hormones and possibly other forces.



(After Kracht [75])

CHART 1

The best experimental material for the study of the principles involved in regulating numbers of cells is at present in the domain of endocrinology. Conditions can be created whereby uncontrolled proliferation of one cell type is obtained, resulting in a tumor-like growth. Manipulations attaining this need not involve any intrinsic alteration in cells causing them to behave as cancer cells.

Recent studies on the induction and transplantation of pituitary tumors have demonstrated that the change which initiates and maintains this neoplasm resides at first in the host and that subsequently modifications occur in both the hosts and neoplastic cells (40, 42). A survey of the literature disclosed that the same sequence of events holds for a large number of neoplasms. In view of the current trend to regard the basic change in cancer as a sudden alteration in cells, it seemed desirable to survey important contributions pointing to a sequence of gradual changes from normal through hyperplasia to conditioned and autonomous growths.

THYROID AND PITUITARY INTERDEPENDENCE IN RELATION TO TUMOR DEVELOPMENT IN THESE ORGANS

The interdependence of thyroid and pituitary can now be studied with great precision by means of quantitative assays of secretions of these endocrine organs. The reciprocal relationship between TH and TSH, well formulated by Stanley and Astwood (121) and confirmed by D'Angelo and Gordon (25, 26), and others, is surveyed diagrammatically as shown in Chart 1. TSH increases the capacity of the thyroid to fix iodine and synthesize TH. The first step is an enzymatic break-up of colloid with release of TH. This finds its expression morphologically in scalloping of the round margin of the colloid in "resting" thyroids, one of the signs of hormonal stimulation in the bioassay of DeRobertis (27), evident as early as ½ hour after administration of TSH. Loss of iodine is utilized in TSH assays in the chick (73) in which iodine loss is followed by iodine capture and its synthesis into TH.

Thyroid tumors.—Interference with thyroid-pituitary-hormonal relationship may result in the development of either pituitary or thyroid tumors. Sustained interference with TH production, as accomplished by goiterogens, causes a lasting increase in the production of TSH, which in turn causes hyperplasia of the thyroid; if sustained, the latter causes the formation of adenomas and, later, carcinomas in this gland (12, 96).

Thiouracil is an ideal antimetabolite for the production of thyroid tumors, because it does not injure the thyroid cells while blocking their ability to synthesize TH and thereby stimulating the pituitary to discharge an increased quantity of TSH. It is the latter which causes the tremendous hyperplasia and adenoma formations of the thyroid gland in thiouracil-treated animals, as it does in normal mice given excessive amounts of TSH or in those bearing (autonomous) pituitary tumors secreting TSH.

The induction of thyroid neoplasms by goiterogens and co-factors was extensively studied by Bielschowsky *et al.* (10–12, 61, 62), Purves and Griesbach (104, 105), Dalton *et al.* (24), Morris *et al.* (96, 97, 131), Goldberg and Chaikoff (51, 53), and Money and Rawson (93). Neoplasms of the thyroid induced by thiouracil can be grafted on mice whose thyroids are similarly blocked with thiouracil, but not on normal mice (11). Such tumors, though dependent on TSH, often metastasize to the lungs (11, 96). Whether or not such tumors and the similar human metastasizing thyroid adenomas are considered neoplastic depends on the definition of a neoplasm. In our terminology

such thyroid tumors are conditioned neoplasms. In the course of subpassages in thiouracil-treated mice the dependent growths give rise to autonomous growths which possess individual features of their own and can be grafted on normal mice (96, 97, 131). A correlation has been demonstrated between the histologic picture of the different tumors and their relative capacity to assimilate iodine and synthesize it into thyroxine.

The morphologic changes in the thyroid of normal mice bearing autonomous pituitary tumors which secrete TSH are identical with those produced by long continued administration of thiouracil. To accomplish this, the pituitary tumors need not be large. Minute, semi-autonomous pituitary tumor grafts, present during several months and barely visible with the naked eye, can cause enormous enlargement of the thyroid with adenoma formations, even though the growth of these grafts is held in check at sites of the grafts.[2] Thyroid adenomas induced by TSH-secreting pituitary tumors are indistinguishable from those induced by thiouracil. They may metastasize to regional lymph nodes, but gross pulmonary metastases have not been noted thus far.

TSH-secreting pituitary tumors.—Radiation injury with large doses of I^{131} ($\sim$ 200 μc. in mice) also abolishes TH production but, in contrast to thiouracil, causes atrophy of the thyroid with stenosing arteritis and fibrosis. Thyroids so injured are incapable of undergoing hyperplasia, either because of damage to their epithelial elements or to the stroma or to both. Deficiency in TH in mice treated with large doses of I^{131} causes a sustained stimulation (or loss of restraint) of the pituitary, terminating in multifocal development of adenomas in the pituitary (40, 42, 52, 55, 56, 108). This theory explains why radiothyroidectomy is likely to produce pituitary tumors, and long continued administration of goiterogens, as thiouracil, is more likely to produce thyroid tumors. Doses of I^{131} which do not destroy the thyroid completely but interfere with its regenerative capacity can also lead to the development of pituitary tumors (56, 108).[2]

This theory implies that surgical thyroidectomy might also induce the development of pituitary tumors. This has been denied by some investigators, but to our knowledge thyroidectomized rats have not been studied long enough for the development of pituitary tumors. In mice surgical thyroidectomy is difficult to perform. Furthermore, it has not been shown that pituitary tumors can be induced in other species of animals. Experiments are now in progress[3] to discover whether surgical thyroidectomy would induce tumors in the C57 strain of mice, highly susceptible to their induction by I^{131}, and whether other strains of mice would respond equally well to radiothyroidectomy with development of pituitary tumors. Thus far such tumors were induced in the two other strains tried (Rf and Ak).

Changes in the pituitary gland of patients with untreated primary hypothyroidism of long duration are not adequately described. Since the discovery, made in 1891, that feeding thyroid will compensate for thyroid deficiency, autopsies on patients who had no functioning thyroid gland for 10–20 years before death are not on record. Focal hyperplasia of "neutrophilic" cells (90) and one case of adenoma have been observed. Patients currently radiothyroidectomized are given thyroid hormones when the symptoms of athyroidism become severe, and, theoretically, this treatment should prevent induction of pituitary tumors.

Species and strain differences in the inductivity of pituitary tumors should be expected. In animals castrated or treated with estrogens, pituitary tumors develop with high frequency in some strains and not in others (49). Strain or genetic differences in response to TSH (5) or to carcinogens in general are well known (Kirschbaum *et al.* [74]).

It is also possible that irradiation of the pituitary incidental to radiothyroidectomy by I^{131} is a factor contributing to the induction of pituitary tumors in mice (57). This, however, does not apply to factors influencing the growth of transplanted pituitary tumors which can be grafted on radiothyroidectomized mice long after disappearance of radioactivity (40, 42). Furthermore, there is a quantitative relationship between thyroid depression and growth of grafted pituitary tumors. Such tumors usually fail to grow in normal mice and in those treated with 25 μc. of I^{131}. In mice whose thyroid function has been moderately depressed by approximately 75 μc., they grow after a long latent period and at a very slow rate. They grow faster and after a shorter latent period in animals whose thyroid is completely destroyed by approximately 200 μc. (41). In these experiments radioiodine is used merely to depress thyroid function, and the tumor grafts are made after I^{131} has disappeared from the body. Thus, the retardation of growth of grafted pituitary tumors is due solely to the inhibiting force of TH.

Induction of pituitary tumors by radiothyroidectomy can be prevented by thyroid grafts or by administration of thyroid hormone (Goldberg and Chaikoff [51], Gorbman [56]); similarly, dependent pituitary tumors are held in check by the administration of desiccated thyroid or thyroxin

[2] J. Furth, W. T. Burnett, Jr., and E. L. Gadsden, unpublished data, this laboratory.

[3] Dr. J. N. Dent, this laboratory.

or thyroid grafts.[2] Radiothyroidectomy is more effective than surgical thyroidectomy in such experiments, because all thyroid cells are inhibited in the former, while residual and aberrant thyroid cells undergo compensatory hyperplasia following incomplete surgical thyroidectomy. Regeneration of irradiated cells which escaped destruction is inhibited in an irradiated field because of the reduced blood supply and extensive scarring. Failure of residual thyroid tissue, surviving partial destruction with I^{131}, to enlarge in response to the administration of thiouracil has been described by Maloof *et al.* (89).

The induction of chromophobe pituitary tumors in two mice treated with thiouracil has been reported by Seifter *et al.* (114). This requires verification, since such tumors occur spontaneously in mice. In an extensive study on the effect of long continued administration of thiouracil in C57 mice, Dalton *et al.* (24) noted the presence of occasional "thyroidectomy-basophils," degranulation of these cells, and a marked decrease in cytoplasm of alpha-cells, but no pituitary tumors. In our experiments, uninterrupted treatment of C57BL mice with thiouracil during a period of 1 year did not cause pre-tumorous changes in the hypophysis, whereas such changes are noted as early as 1 or a few months after radiothyroidectomy. It is conceivable that sustained blockage of the thyroid with thiouracil will occasionally cause the development of pituitary tumors. Thiouracil stimulates the pituitary and augments the TSH output. The question is raised: why does this stimulation not regularly produce pituitary tumors as does radiothyroidectomy? One explanation may be that the thiouracil-treated animals having thyroids receptive to the excessive amount of TSH spend it, while the athyroid animals do not. It is also probable that the blockage of the TH synthesis is not so complete after thiouracil treatment as after radiothyroidectomy. An analysis of this situation calls for quantitative assays of TH and TSH in the course of tumorigenesis by these two procedures. Since the pituitary is subject to sustained stimulation in mice receiving thiouracil, the possibility of induction of pituitary tumors by this anti-TH compound is a reasonable supposition.

Gonadal-pituitary Relationship

Tumor induction by interference with the gonadal-pituitary relationship is not so simple as tumor induction by disturbance of the thyroid-pituitary relationship, because of the capacity of the adrenals to compensate for the lack of gonadal hormones in the absence of the gonads (94, 132). With the background of present knowledge for such work, it might be possible to produce either gonadal tumors or pituitary tumors secreting gonad-stimulating hormones, and study the dependent and autonomous phases of the neoplastic change leading to their development.

Ovarian tumors.—The reciprocal relationship between gonads and hypophysis has been recognized by Moore and Price (95). Biskind and Biskind (14) made use of the inactivation mechanism of hormones of the ovary by the liver (Zondek [135], Golden and Sevringhaus [54]), whereby a sustained stimulation of the pituitary to produce and release gonad-stimulating hormones can be attained. They (14) grafted gonads of castrate animals into the spleen or other areas drained by portal veins, and this led to the development of ovarian tumors in rats (14, 103), mice (80, 81, 44), rabbits (102), and guinea pigs (85).

Failure of growth of intrasplenic ovarian grafts in spleens of castrate animals has been correlated with errors in technic enabling drainage of spleens of ovarian secretions into the general circulation; the ovarian secretions which escape inactivation by the liver raise the blood level of ovarian hormones and depress the production of gonad-stimulating hormones by the pituitary. A conspicuous increase in gonadotrophins in the pituitary of castrate rats has been demonstrated earlier by Engle (33) and Evans *et al.* (34) and in those bearing intrasplenic grafts by Miller and Pfeiffer (92). Stimulation of such grafts by anterior pituitary transplants has been shown by Silberberg *et al.* (117). The tumors induced in castrated male mice have been predominantly granulosa-cell tumors, and those in female mice either mixed granulosa-cell tumors and luteomas or pure luteomas. Injections of gonadal hormones in mice bearing intrasplenic ovarian grafts prevented the development of ovarian tumors. Transplanting one ovary into the spleen of unilateral castrates was likewise ineffective to induce ovarian tumors (13, 48).

Intrahepatic metastases after intrasplenic grafts have been noted on few occasions (80, 44), but these no more signify autonomy than do pulmonary metastases of thyroid nodules in thiouracil-treated animals. Assay for autonomous cells calls for transplantation studies in normal and conditioned hosts. Failure to report on negative findings makes it difficult to evaluate how many of the tumors induced by intrasplenic grafts of ovaries were autonomous and at what stages of this tumorigenesis autonomy was acquired. Granulosa tumors induced in the rat by transplanting ovaries in the spleens of castrate animals were found by Peckham and Greene to be not fully autonomous, as they failed to grow in normal rats (103). Simi-

larly, most attempts to graft such tumors in the subcutaneous tissue of normal related mice failed in our experiments (44), but no systematic parallel transplantations were attempted at these two sites in normal and castrate animals.

Ovarian tumors in irradiated mice.—Ionizing radiations of all types cause the development of ovarian tumors in most female mice exposed to doses larger than about 30 r (43). This carcinogenesis has been likened to that of intrasplenic grafts of ovaries in castrates, and ample evidence has been presented in support of the view that some endocrine "imbalance" is a major factor in the genesis of these tumors (Gardner [48]). The ovarian tumors induced by irradiation are complex; some are composed of cells under pituitary influence (the granulosa and lutein-celled tumors); some are not (as the tubular and papillary adenomas). The latter are not likely to be due directly to a hormonal imbalance. The situation is complicated, and an experimental analysis explaining adequately the induction of tumors in the ovaries by irradiation is still needed. Such primary growths are not readily transplantable, and thus far only a few which proved readily transplantable were studied extensively (4). A direct effect of irradiation on the ovary is indicated by the studies of Kaplan (72), who found that irradiated ovaries implanted into irradiated and nonirradiated spayed mice gave rise to granulosa-cell tumors, luteomas, and related neoplasms, while nonirradiated ovarian grafts in irradiated, spayed animals yielded only one sarcoma that may not have originated in the ovarian tissue. A host factor conditioning the grafts was indicated by lack of tumor development when irradiated ovaries were implanted into nonirradiated, nonovariectomized mice. Ovariectomy alone was sufficient to condition the host. It can be assumed that both gonadectomy and destruction of ovaries by irradiation enhance the production of gonad-stimulating hormones by the pituitary. Intact ovarian endocrine function inhibits the development of tumors in irradiated ovaries, as was already noted by Lick *et al.* (82). The dependent phase of these tumors deserves further investigations. Among the questions to be answered is whether ionizing radiation causes a direct change in the cells of the ovary or merely disorganizes the structure of this organ which is composed of cells with widely differing sensitivity to ionizing radiations and whose growth and differentiation are highly interdependent.

Testicular tumors in intrasplenic grafts of castrates.—In grafts of fragments of testes into spleens of castrate rats, Biskind and Biskind (15) noted a tumor-like nodule composed of granulosa cells. Twombly *et al.* (125), following this lead, produced in numerous animals Leydig cell and teratomatous tumors, the largest of which measured $5 \times 4.5 \times 3.5$ mm. One of these proved transplantable into the spleen of a castrate rat. No tumors were observed in the control rats. The difficulty of inducing similar tumors in mice by this procedure is likely to be technical and not one of principle.

The induction of interstitial-cell tumors of the testis by injection of estrogen (Bonser and Robson [16, 17], Hooker and Pfeiffer [66], Shimkin *et al.*, [115]) is the result of pituitary stimulation and not of a direct effect of estrogens on the testes.

Chromophobe pituitary adenomas induced by estrogens.—Zondek (136) found that large doses of estrin produced chromophobe adenomas in male rats not normally subjected to large amounts of estrogens. The role of genetic factors in the induction of pituitary tumors by estrogens has been shown by Gardner and Strong (45, 49), who found that pituitary tumors occur most frequently in estrogen-treated mice of the C57BL strain (the same in which pituitary tumors are easily induced with radiothyroidectomy) or in hybrids of this strain. In their experience estrogen-treated mice of at least six other strains rarely or never acquire such tumors, which are extremely infrequent in their control mice. The simultaneous injection of androgen will reduce the incidence of such tumors (48). These tumors contain little gonadotrophic hormone, as disclosed by assays in hypophysectomized mice (48). Dunning *et al.* (30) induced pituitary tumors by insertion of diethylstilbestrol pellets in rats of several strains; the Fischer strain proved most susceptible. A large pituitary adenoma so induced proved transplantable in all six rats in which diethylstilbestrol had been implanted previously and attained a diameter of 1.5 cm. This growth "in many respects resembles a neoplasm, but it failed to grow upon transplantation into untreated rats of the same inbred line" (30). Similar tumors induced in mice by estrogens grow subsequent to transplantation in other mice of the strain if the hosts are given estrogen, but do not grow in normal mice, and thus they are not completely autonomous. Once the tumors become established, however, they grow in absence of estrogen treatment (48).

Although evidence was not presented that the adenomas so induced were hormone-secreting, it appears probable that the pituitary tumors occurring in castrates and in estrogen-treated animals were secreting gonad-stimulating hormones. If so, the question arises why the estrogen produced does not inhibit such pituitary tumors from growing.

An answer to this question calls for quantitative assays of gonadal and gonad-stimulating hormones in the course of induction and progressive growth of such tumors. It is possible that the hosts in which such tumors were induced were nonresponsive to the hormones of the tumors. The process appears analogous to that of pituitary tumor induction by radiothyroidectomy. The very lack of a responsive organ appears basic in the development of pituitary tumors. It is interesting to speculate on the kind of gonadal hormones present in these tumors. Would tumors induced by castration contain predominantly FSH (to compensate for the lack of estrogens) and those induced by estrogen treatment LSH (to neutralize the estrogens); or are the two so interrelated that disturbance of one produces profound alterations in both? Gardner assumes that estrogens act on the pituitary gland much as thiourea acts on the thyroid, in that they prevent or reduce the secretion of some but not all of the hormones that the hypophysis normally produces. Under such conditions the gland hypertrophies, and, in the pituitary, adenomas form in animals of some strains (48). Other explanations are possible. To produce a tumor a growth stimulus is needed. Goiterogens interfere with TH synthesis and thereby stimulate the pituitary to produce TSH, and it is the latter which causes the thyroid tumors. Estrogens depress FSH-producing cells and should no more stimulate the pituitary than excessive amounts of TH. The incentive is to stimulate production of hormones which would neutralize the excessive amounts of injected estrogens, and this calls for pituitary cells which secrete lutein- or interstitial-cell-stimulating hormones. Assay of the hormones of tumors induced by estrogens might determine whether any of these suppositions is correct.

Chromophobe adenomas of the pituitary in castrates.—Dickie *et al.* (28) noted the presence of pituitary tumors of this type in many mice of some strains but not of others. No data are available on the rate of induction of such tumors by castration or on hormonal secretions and the character of such tumors. Frequent development of such tumors after gonadectomy would be expected were it not for compensatory gonadal hormone secretions by the adrenals. Cells of normal adrenal cortex secrete only traces of or no gonadal hormones, but, under abnormal conditions, they may synthesize such hormones (94).

Adrenal tumors developing in mice castrated early in life (Woolley [132, 133]) can be regarded as conditioned neoplasms; their development can be prevented by gonadal hormones (Woolley and Little [134]). Cells of the adrenal cortex are stimulated to synthesize gonadal hormones when the gonads are absent, and this stimulus causes hyperplasia followed by neoplasia. Some of these adrenal tumors metastasize and are transplantable to normal hosts (134); thus, as other conditioned neoplasms, they can acquire autonomy.

Interstitial-celled testicular tumors induced by estrogens.—Leydig-cell tumors can be induced by prolonged treatment with estrogens in mice of A and JK strains (46). These tumors grow when transplanted into mice of the same strain, provided the hosts receive estrogens (47). They have grown in untreated males but seldom in untreated females. If, however, estrogen is administered after the transplant has remained dormant for a period of several months, the grafts begin to grow (Gardner [47]). Similar results were obtained by us with TSH-secreting conditioned pituitary tumors which, when grafted on normal hosts, remain dormant for several months after grafting but can be "activated" by the destruction of the thyroid. The ability of tumor cells to remain dormant in the skin has been described by several investigators (107, 111). Estrogens or their metabolites increase the production of luteinizing hormone (LH) which is similar to or identical with the stimulating hormone of the interstitial cells. The excessive amount of LH is responsible for the accelerated growth rate of these tumors in the course of estrogen treatment.

Mammary Gland Tumors

The hormonal relationship between the mammary gland and ovary has long been recognized and applied to control of mammary gland tumors. Oophorectomy in control of breast cancer was suggested in 1889 by Schinzinger (113), performed in 1896 by Beatson (6), and was found of value in young women. With the advent of x-rays the same effect was sought by ovarian irradiation, and this procedure is still being advocated (Douglas [29]). Testosterone was used later with the idea of neutralizing estrogens (Ulrich [127]); and still later large doses of estrogens were advocated. Both depress production of gonad-stimulating hormones of the pituitary which is the opposite of surgical castration. If estrogens are the stimulants of the mammary tumors, their use would seem illogical, but regressive effects after estrogen in certain cases of mammary cancer has been proved. If, however, estrogens would depress other hormones which influence the growth of breast tumors, the usefulness of estrogens in the treatment of mammary tumors might be explained. This problem has not been fully analyzed experimentally. Huggins and Bergenstal (68) demonstrated that adrenalectomy can

often cause a regression of mammary cancer in women who do not possess ovaries. Estrogen excretion in oophorectomized women is often considerable; in these subjects it is no longer detectable by bioassay subject to adrenalectomy (personal communication). The action and the levels of the three hormones known to affect the normal breast are not well known in relation to development and growth of breast tumors.

The behavior of the experimental pituitary tumors may furnish an explanation for the paradoxical response of human breast tumors to either androgens or estrogens. At first these pituitary tumors are inhibited by TH. Later they gain autonomy and can be grafted in normal animals whose thyroids, stimulated by the TSH of the grafted tumors, secrete excessive amounts of TH. Such autonomous tumors are not inhibited by TH; just the reverse, they seem somewhat stimulated by TH and depressed by lack of it. The situation is similar to that observed in the course of experimental therapy with antimetabolites: cancer cells which at first are inhibited by antimetabolites may acquire resistance to them and later may be even stimulated by them (Law [79]).

Induction of pituitary tumors is readily prevented by correction of the endocrine defect which induces these tumors, and a similar treatment may restrain the tumors. But hormones administered to tumor-bearing hosts merely affect growth rates and do not directly destroy grafted cells in a genetically compatible environment. Hormonal control of mammary gland tumors in man usually is transient, but may persist for 5 or more years. Such tumors, while responsive to hormones which may have played a role in their induction, are not destroyed by them. The grafts survive even though their size is microscopic. Secondary adaptative changes may occur in both host and the grafted cells by which the host tends to restore the balance altered by introduction of hormones, and the cells acquire greater and greater autonomy.

The causation of mammary gland tumors of mice by estrogenic stimulation (Loeb [86, 87]), Cori [22], Murray [100], Lacassagne and Nyka [77]) is the first documented example of cancer induction by a normal physiological stimulant of an organ. The morphological sequence of changes occurring in the course of induction of mammary tumors in mice by estrogens is well described by Burrows (19). Simple hyperplasia is followed by adenoma formations terminating in carcinoma. The existence of a responsive phase in the evolution of mammary gland tumors of mice was well described by Foulds (35, 36), who observed an enhanced growth of breast tumors during pregnancy with regression after parturition. Foulds pointed out that in mice the growth of some mammary tumors occurs in waves with peaks at or near parturition; during the interval the tumors remain in abeyance but recur promptly when the mouse becomes pregnant again.

While in mice sustained estrogenic stimulation terminates in development of carcinoma, in rats the usual change is a benign fibroadenoma, although administration of large doses of estrogens will cause carcinoma of the mammary gland also in this species (Geschickter [50]). As early as 1919, Leo Loeb recognized that, unlike most autonomous malignant tumors of the breast, benign tumors of this organ are highly susceptible to the same proliferative hormonal stimulation as normal breast (87). The fibroadenomas of the rat have but a minimal intrinsic and a high grade of responsive growth force in terms of Foulds. When grafted on genetically compatible hosts, they grow very slowly; their propagation is more successful in females than in males (87). Heiman and Krehbiel (63, 64) found that castration of female rats reduces and that of the males increases transplantability of fibroadenomas, and that estrogens and gonad-stimulating hormones stimulate the epithelial elements of such growths. By injection of estrogens for 50 days or longer, precancerous changes were produced in these fibroadenomas (87). Androgens and progesterone inhibit the epithelial elements and stimulate the fibrous stroma and may drive the latter to form sarcoma-like growths (63, 64).

The temporary retardation of mammary tumors by estrogen or androgen treatments in women and the effect of these hormones on breast tumors of mice and rats suggest that these growths are not fully autonomous. At an earlier period, they might perhaps be fully dependent on some hormonal stimulus.

Prostate and Gonadal Hormones

Huggins and Hodges (69) demonstrated that hormones which normally control prostatic function will also control growth of carcinoma of the prostate. This tumor is an example of a growth in man with a spectrum ranging from conditioned to highly autonomous type. The cases of Huggins that were controlled by castration (that is, removal of sources of androgens) may be regarded as dependent; those which partially or temporarily regressed after castration or estrogen treatment, as partially dependent; those not influenced by such therapy, as autonomous. The beneficial effect of castration wears off with time mainly because of the functional metaplasia of cells of the adrenal cortex, resulting in the ability to synthesize gonad-

al hormones. This was soon recognized by Huggins; since the discovery that cortisone maintains life following adrenalectomy, he has been able to remove both gonads and adrenals and cause a more lasting regression of prostatic carcinoma than was accomplished by removal of gonads alone (67, 68).

Conditioned Neoplasms of Other Organs

Skin.—The sequence of changes in the skin in the course of carcinogenesis has been subjected to close scrutiny (7, 8, 107, 111). Phases in cutaneous carcinogenesis have been demonstrated by these investigators and others cited by them. Both stages of tumor growth analyzed by Rous *et al.*, named "initiation" and "promotion," are believed to concern the autonomous phase; the first refers to transformation of normal cells into malignant cells, the latter to promotion of their growth. Promotion by carcinogens is termed by Berenblum epi-carcinogenesis. Berenblum (7, 8), Rous *et al.* (37, 107), and several other investigators (98, 99, 109, 116) have shown that promotion can be brought about by noncarcinogens. Agents that stimulate mitotic activity naturally enhance the action of those carcinogens which are more likely to affect cells in mitotic division than resting cells (43). Neoplastic cells can remain dormant for years until some proliferative stimulus, which in itself is not carcinogenic, causes them to proliferate and form tumors (9, 37, 111, 130). Hyperplasia of mouse skin after short treatment with benzpyrene may subside and the skin become normal if treatment is discontinued after 5–10 weeks (112). There is no way of telling whether these latent cells are autonomous or dependent; both can, to some extent, respond to proliferative stimuli or restraints; the second treatment may primarily affect either the neoplastic cell or its environment. Even metastasizing malignant cells are restrained at some sites. Regression of hyperplasia is a common phenomenon; the problem under consideration is that of distinguishing between hyperplasia and neoplasia. Is it not possible that the "initiation" resulting in formation of latent neoplastic cells is due sometimes not to an alteration in these cells but to that of the locally restraining forces and that the tumor cells formed are dependent as are the pituitary tumor cells in radiothyroidectomized mice?

The possibility of conditioned hemoblastosis (leukemia).—In view of the general acceptance of the theory also advocated earlier by this reviewer that the basic alteration in leukemia resides in the hemopoietic cell, this theory will now be reconsidered. While the regulatory mechanism of the number of cells is best known in the domain of endocrine glands, excessive formation of blood cells, as that of other cells, is probably under some restraint, and mere lack of such a restraining factor or a blocking force may conceivably cause leukemia. In the experimental mammalian leukemias studied thus far, the leukemic cells possess autonomy; when grafted in normal hosts the normal restraining forces do not limit their proliferation. However, the basic change may in some cases of leukemia reside in the host. All gradations may conceivably exist between the two extremes: leukemia with seemingly normal cells and leukemia with fully autonomous cells. Of the many types of leukemias seen in man, only that type has been studied in animals in which an immature blood cell is so changed that it is capable of overcoming all regulatory forces of the normal host and proliferating until death of the host. These are examples in which the leukemic cells possess maximum autonomy. In some human leukemias, in which maturation of the primitive hemopoietic cells is conspicuous and the course of the disease is chronic, the leukemic cells are immature but not altered and may lack autonomy. Such leukemias do occur in animals but have not been adequately studied experimentally (39).

Other organs.—The distinction between dependent and autonomous tumors has been clearly recognized by many investigators. In a recent article Büngeler (18) discusses the relation of dependent growth disturbances to tumors. In his opinion dependence indicates that a growth is not a "true" tumor: "Diese Abhängigkeit der geschwulstartigen Hyperplasien von übergeordneten Regulationen, von hormonalen und neuralen Einflüssen sowie von Stoffwechselstörungen zeigt dass es sich nicht um echte Geschwülste im Sinne der allgemein anerkannten Geschwulst-definition handelt." Among the possibly conditioned growths discussed by Büngeler are: adenomas of the thyroid, prostate, breast, uterine mucosa and musculature, adenomas of the parathyroid associated with osteitis fibrosa, adenomas of adrenal cortex with hypertension and Cushing's Syndrome, struma of the glomus caroticum, cylindromas of the salivary gland and carcinoids of the respiratory and digestive tract, multiple myeloma, the driving force of which may be a disturbance in globulin synthesis; the leiomyomata of the gastrointestinal tract, which may be secondary to neurogenic disturbances. Abdominal fibroids induced in guinea pigs by estrogen treatment are probably dependent growths (83). The parathyroid adenomas have also been related to a pituitary dysfunction (130), but there is no good evidence that the parathyroid function is under pituitary control. Earlier views

that the hyperplasia of the parathyroid caused by a disturbance of the metabolism of calcium and phosphorus predisposes to tumor formation are not supported by evidence (130). Nevertheless, the older views relating parathyroid growth to antecedent changes in calcium-phosphorus metabolism deserve further consideration in the light of the experiments reviewed here. Andervont and Dunn (3) recorded the inability of many primary hepatomas to become established or be serially transplantable in genetically compatible hosts. The growth of these hepatomas can therefore be considered as dependent on some factors residing in the hosts.

Schematic Tabulation of Developmental Phases of Some Well Studied Neoplasms

Table 1 is a tabulation of examples in experimental carcinogenesis in which "step-like" changes were studied, with data which suggest the possibility of the existence of conditioned and autonomous stages. This is compiled for didactic purposes only and is far from complete. The events which take place are not known well enough and are not identical in different organs, but the underlying principles and the trend appear to be the same in all. For further information on "conditional neoplasms and threshold neoplastic states" in skin the reader is referred to an article with this title by Rous and Kidd (107), on latent neoplastic states to those of MacKenzie and Rous (88), Berenblum and Shubik (9), and the papers cited by them. The concept of "initiation" and "realization" was evolved in studies of cutaneous carcinogenesis; that of "responsive" and "intrinsic" growth (Foulds [35, 36]) was arrived at in studies of mammary tumors; the importance of cocarcinogenesis was pointed out by Lacassagne (76), Berenblum (7, 8), and studied by numerous investigators (38, 43, 98, 99, 109, 118, etc.) though not to the extent of its presumed importance. The three periods of carcinogenesis (induction, reversibility, and progression) in the scheme of Rusch (110) require better understanding in the light of recent knowledge of host factors contributing to the progression of neoplastic growth.

TABLE 1

Schematic Tabulation of Conditioned and Autonomous Neoplasms

Organ	Initiating and promoting agents	Precancerous change	Growth		Species best studied
			Conditioned or responsive ("Initiation")	Autonomous or unresponsive ("Realization")	
Skin	Tar and other chemical carcinogens, virus, ultraviolet rays, ionizing radiations	"Dermatitis"	Wart	Squamous carcinoma	Rabbit Mouse
Breast	Virus, estrogens, and other hormones, milk stagnation	"Chronic cystic mastitis," hyperplasia	Fibroadenoma*	Carcinoma or sarcoma	Mouse Rat
Ovary	FSH, LSH (transplantation in spleen), ionizing radiations	Regenerative changes of granulosa and other cells	Granulosa tumor, luteoma, adenoma	Same, malignant	Mouse Rat Rabbit
Thyroid	Goiterogens, TSH, acetylaminofluorene	Goiter	Adenoma	Carcinoma	Mouse Rat
Pituitary	Estrogens, gonadectomy, radiothyroidectomy	"Castration" and "thyroidectomy" cells	Adenoma	Carcinoma	Mouse Rat
Bladder	β-naphthylamine, benzidine, acetylaminofluorene	Hyperplasia, cystitis	Papilloma	Carcinoma	Dog Mouse

* Also some carcinomas of this and other organs can be conditioned.

According to a widely held concept, benign growths are not "true" neoplasms, because they lack autonomy ("Eigengesetzlichkeit") and owe their existence to adaptation to some stimuli, while the change in "true" tumors resides in the cell. According to Büngeler (18), there are no transitions between the two types of growth. Most of the benign tumors enumerated have not been studied experimentally, and so this list offers opportunities for research on dependent growths. The conclusions derived from observations on these human neoplasms require a revision in the light of recent experimental studies. Multiplicity of a tumor of the same type is a frequent attribute of dependent neoplasms, but it is no proof of their character; similar malignant neoplasms induced by carcinogens are also frequently multicentric. The apparent "fluid" transitions between dependent and autonomous neoplasms have been amply documented, although it is left to future research to determine whether the transition is truly "fluid" or

DISCUSSION

Modification in cells.—In the embryo organizers set the process of dependent differentiation (Needham [101]). In the adult "maturation factors" set the development toward the presumptive fate of cells. Failure of differentiation (e.g., a myeloblast or myelocyte into a mature granulocyte or basal cells into squamous cells) is a feature of many neoplastic cells incidental and related to but not identical with the basic neoplastic change affecting cells. In absence of folic acid, a specific maturation factor, megaloblasts will proliferate and fail to mature, but this does not result in tumor-like formations. A neoplasm may result from (*a*) lack of the normal restraining force of cells in the presence of favorable nutrients or (*b*) a change in the reactivity of the cell to normal growth stimuli, resulting in an escape from responsiveness. The latter is analogous to a change in competence in terms of embryologists. Whether this change is brought about by abnormal differentiation resulting in an escape from the normal controlling field or a somatic mutation (Lockhart-Mummery) will not be discussed here (Needham [101] and others [38, 60]).

A concept of an organ-specific growth control was proposed by Paul Weiss (128, 129) as follows: Protoplasm synthesis of a given organ yields (*a*) "templates" for further reproduction and (*b*) accessory diffusible compounds capable of inactivating the former. As the latter, accumulating in the common humoral pool, reach critical concentration, growth ceases. Partial removal of an organ by reducing the concentration of (*b*) will entail compensatory growth in the rest of the organ. In experiments aimed to support this theory Weiss *et al.* found that in tissue culture the presence of a given organ extract markedly reduces capacity for differentiation in the homologous organ.

The view that the cancerous change is that of abnormal differentiation appeals to embryologists, while geneticists explain the cancerous change by a somatic mutation. The hereditary matter (genes) carried in the chromosomes are the self-perpetuating substances composed predominantly of nucleic acids. Accordingly, the basic change is considered to be an alteration in this chemical, producing abnormal genes and so perpetuating the abnormal cell. These two views (abnormal differentiation and somatic mutation) are not mutually exclusive.

Genetic studies have been conducted with tumor cells transplantable in normal hosts; therefore, they are concerned only with the nature of modification of cells of autonomous neoplasms. If transplantation patterns go hand-in-hand with genetic differences, transplantation studies may be considered to indicate that a somatic mutation is the basic change in autonomous neoplasms (Strong [122], Tatum [124], and Snell [120]). The evidence collected in this review indicates that such changes are not necessary in order that cells may grow with no apparent restraint and that at the beginning of the development of many neoplasms the change may reside in the host and not in the cell.

Acquisition of autonomy.—The nature of the change occurring while cells acquire autonomy has been the subject of decades of discussion (Loeb [87], Rous and Kidd [107], and others). Acquisition of autonomy goes with a permanent modification in cells; it is maintained through subpassages in normal hosts and is not known to revert to dependency. It is not a rigidly fixed change, as it tends to gain momentum in the course of transplantation. Increase in proliferative vigor is common to most transplantable tumors and leukemia, and clinical observations point to a similar event in the course of spontaneous neoplasms. These statements require some qualifications: if a few cells regress or revert to normal type, they would not be noted under current methods of propagation. Interesting examples of regression of tumors in the course of experimental carcinogenesis are mentioned by Rous and Kidd (107) and Rusch and Kline (111). Among the human neoplasms known to be capable of regression are chorionepitheliomas, juvenile papillomas of the larynx, leiomyomas of the uterus, and neurogenic tumors of the newborn. There are factors other than physiological growth-restraining forces which can cause regression of a tumor (112).

Whether the changes occurring in the neoplastic cells are sudden or gradual is debatable. It is possible that sudden changes occur in some cells whereby the population becomes mixed and those with greater proliferative vigor and autonomy gradually outgrow those with lesser vigor and invasiveness. These two possibilities are not mutually exclusive; both sudden and gradual changes are conceivable. Mutations are sudden permanent modifications affecting genes, while the observed changes in tumors appear to be gradual ("fluid"). The suddenness of acquisition of autonomy may not be disclosed by the technics employed for its detection. Mutations never affect an entire population, and if only a few cells mutate, the mutants may be masked and outgrown by the rest of the successive population. An assay of autonomy calls for grafting single tumor cells on normal genetically compatible hosts. Failure of growth in normal hosts in the presence of good growth in conditioned hosts would indicate lack of autonomy. An experimental analysis of this situation calls for progeny

assays of cells as done with unicellular organisms. This is now feasible with cancer cells and is being done by means of tissue cultures (32) and with "ascites" tumor cells (Hauschka).[4] The experimental evidence surveyed by Law (78) favors the assumption that the variant cells arise by spontaneous mutation which occurs constantly in a population of cells, the metabolic antagonists acting as a selective agent in the propagation of the variant forms.

A progeny test of neoplastic cells of vertebrates will prove to be far more intricate than that of unicellular organisms. In the course of our studies of dependent pituitary tumors the grafts in normal hosts ranged in size from a few millimeters to several centimeters. In numerous animals the grafts were not identified at autopsy made 1 year after grafting. The minute "takes" would have been missed entirely were it not for the presence of the greatly enlarged thyroid glands indicative of hypersecretion of TSH, and this called for a microscopic search for the grafted tissue. In genetically compatible hosts neither normal nor altered pituitary cells perish when grafted; the grafts will "take." Normal cells are expected to proliferate and function to the extent called for by the respective homeostatic mechanism. A question to be answered is why such grafts, smaller in size than a normal pituitary, should cause hyperthyroidism 1 year after grafting. If the cells are normal, the thyroid-pituitary regulatory mechanism should prevent hypersecretion; if they are altered, why the lack or slowness of proliferation?

The changes which normal cells are capable of undergoing to free themselves from normally restraining forces and the even more remarkable later changes to acquire dependency on that force recall similar phenomenona affecting bacteria exposed to antibiotics (91) and neoplastic cells exposed to carcinolytic agents (78, 79). Ability to acquire resistance to or dependency on injurious or inhibitory agents appears to be a biological phenomenon common to unicellular organisms and to such cells of a multicellular organism that are capable of independent existence. The mechanism and nature of these modifications, commonly referred to as mutations, are subject to extensive current research.

Responsive and intrinsic growth rates.—Foulds conceives the total growth of cells as the sum of the intrinsic and responsive growth rates. All cells which can give rise to cancer possess the ability to multiply at a given rate, provided the environmental conditions are constant. They also have the capacity to respond to nutritional and hormonal growth factors, temperature, pH, etc. The intrinsic growth rate of normal cells is in general low; their responsive growth rate is high. The cancerous change goes with acquisition of a greater intrinsic growth rate and diminished responsiveness; the more malignant a cell, the greater the intrinsic and the less the responsive growth.

This concept describes the changes in cells in the course of acquisition of autonomy, even though it does not define the character of these changes and fails to account for dependent growths in which the change is in the host. It is a meritorious concept to be pursued further. Dependent growths are responsive, and their relative growth rates can be analyzed further in terms of responsive and intrinsic growth. With acquisition of autonomy some responsiveness is maintained, and assays for the kind and degree of responsiveness will aid in characterization of the neoplastic cell.

Complexities of tumorigenesis.—(*a*) Explaining tumorigenesis by disturbances of a "hormonal imbalance" is an over-simplification of the problem. How does lack of a hormone (e.g., TH) stimulate the pituitary? As concerns induction of ovarian tumors by grafting ovaries in the spleen of castrate animals, it has been suggested (1, 119) that oxidative degradation products of ovarian steroids in the liver are the stimulants of the pituitary, and not the low level of estrogens in the blood. It is still debatable whether ovarian growth is regulated through stimulation by gonadotrophins or inhibition by estrogens or their metabolites (65, 71).

b) Ionizing radiation in doses above approximately 50 r causes the development of ovarian tumors in almost every female mouse. If this were due solely to a hormonal imbalance, why should such an imbalance occur when all types of hormone-secreting cells of the ovary are present and could come to an equilibrium with the pituitary? In rats and rabbits ovarian tumors were induced by intrasplenic grafts but not by ionizing irradiation. Furthermore, some of the ovarian tumors induced (e.g., tubular adenomas) are composed of cells that are not known to secrete hormones or to be under hormonal control. If ionizing radiations cause "neoplastic" mutations in cells, why the lack of correlation between latency period and rate in induction and the quantity of ionizing radiations?

c) Bagg demonstrated experimentally that stagnation of milk enhances the likelihood of a cancerous (autonomous) transformation of cells of the mammary gland (cf. 86). On this basis, an endogenous carcinogen was postulated in the re-

[4] T. S. Hauschka, presented at the Transplantation Conference held by the National Cancer Institute, 1952; to be published.

tained secretions during the course of development of both spontaneous breast carcinoma and that induced by continued estrogen treatments. The ability of estrogens to produce a variety of neoplasms including leukemia is remarkable; the mechanism by which this is accomplished is obscure. Control of a neoplasm by a physiological agent suggests the possibility but is no absolute proof of a causal relationship between the two (cf. 70).

d) In tumorigenesis by hormones the following variables require consideration (48): rate of production of the hormone, rate of its destruction, capacity of end organ to react, and quality of hormone produced, along with other general variables including genetic influences, age, and nutritional state. These factors have been amply discussed by Gardner and others cited already. The role of nutritional factors is indicated by the studies of Tannenbaum (123) and others.

e) Virus-like agents (31) may cause a neoplastic change by several mechanisms. They may merely stimulate cells to proliferation, and so cause a conditioned neoplasm. Incidental to an increased rate of multiplication, there is an increase in the number of mutations, perhaps also of the mutation rate. Natural selection of cells evading the restraining forces of the host may follow, and so the papillomas, in which the maintaining force is the virus, may give rise to carcinomas in which viruses may be mere "passengers" or may be entirely absent.

f) The term "dependent" has been used in a somewhat different sense by those who have studied induction of skin tumors by viruses or carcinogenic chemicals. In their terminology "dependency" implies dependency on continued exposure to the inciting unphysiological agent. In experiments with endocrine tumors dependency implies deficiency of the physiological restraining forces shifting the balance to the normal stimulating forces. It is possible that some extrinsic agents produce a conditioned neoplasm not by altering cells but by an action of growth-restraining mechanism of the host.

Dependent and autonomous phases in the development of cancer have been also recognized by Greene (58, 59): Autonomous tumors in Greene's experience possess the ability to grow in the anterior chamber of the eye of an alien host while dependent tumors do not. While this definition may be highly arbitrary, it is remarkable that this technic will distinguish in many instances between growths that are judged autonomous or dependent on the basis of the fate of grafts in the subcutaneous or muscle tissue of genetically compatible animals. With acquisition of autonomy goes the ability of an enhanced stroma-inducing ability as judged by growth in the anterior chamber (59) or in the homologous host.[2]

It is important to identify tumor cells in chemical, metabolic, cytologic and other studies as dependent or autonomous (59); the problem is how to determine the states of dependency and autonomy. A malignant growth is conceived by Greene as synonymous with an autonomous growth. Lymphoblastic leukemias and lymphosarcomas that are graftable to every normal genetically compatible host do not produce tumors in the anterior chamber of the eye of an alien host; nevertheless they answer our criteria of autonomous neoplasms. The studies here surveyed indicate that dependent growths (as judged by our criteria) can be just as progressive and fatal as autonomous growths. The ideas correlating acquisition of autonomy with loss of ability to induce antibody production (59) are not supported by our observations. Evidence is lacking that dependent tumors are restrained by antibodies in a natural host; in fact, dependent tumors grow because they are not restrained. On the other hand, it is possible that retrogressive changes and fibrosis in autonomous tumors are due to some antigenic difference between host and grafted cells. In the anterior chamber of an alien host survival of a graft may depend on failure to produce antibodies while the grafted tissue becomes established. Antibody production against tumor cells in their original hosts is yet to be demonstrated.

g) The possible role ionizing irradiation plays in inducing pituitary tumors is currently under investigation. Gorbman and Edelmann (57) found that pituitary tumors do not develop in mice after radiothyroidectomy with small amounts of I^{131} (30 μc.) in animals kept on low iodine diet even though this dose completely destroys the thyroid. However, the same dose of I^{131} will induce pituitary tumors if the animals are given 575 r of x-rays 7 days following I^{131} treatment. Iodine-free diet greatly enhances the I^{131} uptake by the thyroid. Thus, by the use of such a diet, it is possible to reduce the total body irradiation with I^{131} while administering to the thyroids the same destructive dose. The pituitary dose from the I^{131} deposited in the thyroid is not reduced by this procedure. If irradiation contributes to the induction of pituitrary tumors, injury to an organ other than the thyroid and pituitary should be thought of. The gonads, notably the ovaries, are radiosensitive, and irradiation with doses as small as 25 r causes the development of ovarian tumors. It has been assumed that secondary stimulation of the pituitary plays an important role in the induction of ovarian tumors by

ionizing irradiation. If this assumption is correct, a small dose of x-rays combined with 30 μc. of I^{131} will induce pituitary tumors, provided the ovaries are irradiated. It is true that radiothyroidectomy is a specific stimulant to the TSH-producing cells of the pituitary, while ovarian depression to that of FSH- and LSH-producing cells; but there may be some relationship between the two. Pituitary tumors are known to occur in x-rayed animals; the pituitary tumors induced by gonadal dysfunction are chromophobes as are the tumors induced by radiothyroidectomy. Both are considered related to basophiles which, according to current opinion, are the secretors of both gonadotropic and thyrotropic hormones.

The sequence of changes in the evolution of a totally uncontrollable cancer was presented in stages; the stages were given names for didactic purposes. The terms, "proliferative or growth energy" or "growth momentum," "adaptation," and "realization" are descriptive of events as we perceive them. The underlying experiments and those they have stimulated revealed a great deal on carcinogenic processes, but these terms should not cover up our ignorance of the many problems yet to be solved, such as: what determines the growth rate, what is the nature of modification when a cell acquires an enhanced growth rate, what happens in the cell when it adapts itself to a new situation, what is the material basis of the difference between a cell and a host with respect to transplantability of cells at different locations, and, most important, what are the forces restraining proliferation of most cells in a complex organism and what is the mode of action of these hypothetical agents?

SUMMARY

Ideas and investigations are surveyed concerning the stages in the development of neoplasms. It is recognized that alterations in either the host-environment or in the cells can give rise to neoplasms. The former, named conditioned neoplasms, are known to be readily preventable and reversible; the latter, named autonomous neoplasms, are not. Dependency and autonomy are recognized as relative and quantitative terms. Autonomy is never certain. Many autonomous growths pass through a phase of dependent growth, and the usual trend is toward gradual acquisition of greater autonomy. This sequence of events urges greater attention to the study of normal growth-regulating mechanisms and of the very early changes in the evolution of neoplasms. The understanding of these events may ultimately lead to the prophylaxis of some cancers and to their control at their early phases of development.

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Highways and Byways of Cancer Research*

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Most of our Presidents have chosen as the highway, along which their presidential addresses have led, a review of the surrounding countryside in which they have been particularly interested and in which they have unearthed facts and created ideas and concepts. But from time to time a few have strayed into the byways—or what to some seemed byways at the time, but which subsequently proved to be only detours around particularly rough spots along the main highway. It often happens that what seems a byway turns out later to be one of the main highways.

Some Presidents spoke thus, no doubt, because a particular subject intrigued them more than the experimental work which they had done. Some prepared no review of their work or the work of others, possibly because they did not care to take pleasure away from those of the audience who might wish to review the subject on their own. Others took to the byways because they had ceased determining mitotic indices, or distinguishing between tumors and inflammation, or reading the manometers in Warburg apparatus, or looking at curves on a photographic plate from an electrophoresis set-up, or shifting a hydroxyl group—or any one or more of dozens of procedures. I am one of the latter class, for hardly an hour a week is the cover off my beloved microscope. I call it "beloved" because I often try to think of an instrument that has done more for the benefit of us human beings, and none seems to compare.

I am sometimes called an "Administrator"—officially, Director and Scientific Director—and, therefore, if a presidential address may follow the highways and/or byways traversed by the incumbent whom you have honored with the office, I'll choose to speak not of the details of experiments but of other terrain.

The best administrator, I believe, is one who has practiced a science, has therefore specialized in some field and in all probability become adept in the use of particular instruments. And, no doubt, he has worked in close touch with others, perhaps as a member of a group, and has thus learned and appreciated teamwork. These are among the requirements which helped make him a present-day scientist, but his thinking should have covered a wider range. Relations and syntheses are needed as a kind of circulatory system to give life, meaning, and usefulness to the facts which our senses and instruments dig out for us. There is a growing feeling in some quarters that we have amassed too many facts and have thought about them too little—but we cannot go too far and neglect facts. Many pieces of apparatus are so delicate and complicated that specialization in their use is necessary, but thinking need not be fenced within the bounds of an instrument or two. Technical skills without depth and imagination are sterile in research, or in stronger language:

"Technique!
The very word is like the shriek of outraged art.
The mighty have no theory of technique!"

Science is not all induction; it is in part deduction too. The prejudices of the Baconians still hang around, and Mr. Gradgrind has his modern counterparts. Where is there a field broader than cancer research, with relations to be found among the facts of so many disciplines?

This is part of the very great art of science—the construction of syntheses and relations. Billroth, a very great artist in his science and a fine musician, on the evidence of no less than Johannes Brahms, said with conviction:

"Art and Science are twins, and the mother of both is Imagination."

An administrator is fortunate because he can, indeed must, take a bird's-eye view of what is going on, not only in his own institution but in others as well. He performs chores which the experimentalists take for granted have been and will be done; they shouldn't have to think of such things, they have enough to do. Therefore, they have an administrator to help them. There, it's out—to help the experimentalists in their work! This is the immediate and perhaps the only excuse for having administrators. Indeed, in nonprofit

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corporations, such as those in which most of us work, the real reason for having administrators is this, and no other. I may say, parenthetically, that this applies not only to the administrator, but also to Boards of Trustees of individual institutions or of foundations, whose main job (I take it) is to help those in pursuit of the immediate technical details.

The nonprofit corporation is a peculiar institution, entirely different from a business owned by bond- and stockholders. No individual or group owns a nonprofit corporation, and yet everyone owns it, for its capital and operating expenses come in whole or in part from public funds. The citizens, rich and poor, young and old, own it. Therefore, it is to them that those working in any capacity in nonprofit corporations owe their responsibility.

The administrator no longer takes off his coat when he arrives in the laboratory, rolls up his sleeves, and says to himself, "Now, today I will see what happened to the mice that I injected yesterday." Instead, he goes to his office and is confronted with many papers; his appointment book reminds him of committee meetings, of luncheon engagements, of conferences with many people (including the good, and sometimes not so good, folk who have discovered the cause and cure of cancer), of speeches to the laity, of public-relations maneuvers. Always, of course, there lurks the tiny question in his mind: "Can I get a little more money for this project, or another technician for that, or a new and improved piece of apparatus needed in one of the departments?" Or, a larger question, "Where can I get the money to spend money?" Research has many indirect expenses, and, unless they are covered, direct expenses cannot be expended—profitably. Or, the tiny question becomes still larger: "Where can I get funds for exploratory work? One of us is pregnant with an exciting hypothesis that should go to full term, and I'd like to be present at its birth and see what the offspring will be like."

Around this subject I could write a whole, perhaps several addresses. The adjectives "pure" and "applied" are fashionable today and are used as though there were two sharply divided kinds of research. We all know what we mean when we use these words, but to no two of us do they mean quite the same. I find difficulty in constructing just the right paragraphs to explain them to lawyers, bankers, the public generally. So I use the word "exploratory," meaning research into a field in which there are no tangible relations to build on, nothing to invent, nothing to engineer, no highways, no byways, not even a trail. I find I receive more understanding by using this word. To us here it can be put quite simply: there's not enough known to file a project grant application.

I said the administrator, and of course the experimentalist too, ask the question, "Where can I get funds for exploratory work?" Sources are hard to find for many reasons. I wonder if the difficulties have been increased during the past years because the public is so much more familiar with the inventions that are put to daily use. It does not appreciate that they are developed from laborious, long-extended exploratory work of countless patient discoverers of the past. Perhaps it would help if, in addition to the glamour made to surround the "miracle drugs," the "atom bomb," the home in which electricity or atomic power does all the work, and many other such things, our science writers would add a paragraph or two describing a few of the discoveries that were needed and how long it took before the miracle drug could be put to use, and who discovered what. The names and the labors of these patient workers are at least as important as the names of politicians, generals, admirals, and others.

Science has a solid center of accumulated knowledge and a growing edge of new knowledge. It is just beyond this edge that exploratory work needs to be done. It is at the edges that an abundant harvest can be expected, but usually not in a hurry. In cancer we have a solid core of information, but the boundaries are hazy, with the center itself but poorly defined. Naturally, in any science when new methods are developed to further help our senses, the accepted core is re-examined. A revolution may occur. In cancer more discoveries are needed, and I can't say I'd mind a revolution!

The budget is always with us. As it travels along the highway of cancer research, to continue the metaphor, its wheels never soothe with a steady harmonious hum, but change their tune only from squeak to shriek. You experimentalists know why the squeak is never completely silenced. The wise administrator says: "Never confuse budget with income, nor expenditures with—Don't forget the philosophy of Mr. Micawber: 'income one pound, ten, expenditures one pound, nine—happiness; expenditures one pound, eleven—disaster.' " Lucky indeed is the scientific director who has with him a good and understanding administrative director to help in these matters and many others.

Please note that I keep returning to administrators because many of you, like me, have come to that; and I suspect many more of you will eventually arrive at that position. With malice

toward all it is stated that when you have demonstrated your ability in the laboratory for a few years, you can look forward to becoming an administrator.

There are advantages in being an administrator. One sees and hears at first hand from his colleagues of the activities of his own institution. But why the administrator alone? It must be embarrassing to any member of the staff when someone outside the institution enthuses over a recent piece of work by one of his colleagues and the insider knows much less of it than the outsider. At its best, this is poor scientific teamwork. At its worst, it is poor internal public relations.

The administrator acts as liaison officer between the various members of the staff, he sees their needs, and helps relieve them of routine and special worries. Above all, if he is constituted properly, he is a source of continuous encouragement along both the highways and the byways of their work, and he can, if he will, take sincere pleasure in the accomplishments of his colleagues and rejoice with them in their discoveries. He is one of the main interpreters of the plans, the results, and the implications of the experiments in his institution, to his confreres in his own specialty, in the branches of clinical medicine, to his Board, and to the public generally. This is a challenge to high skill and worthy of anyone's effort.

In the repeated evaluation of experimental programs the administrator plays a delicate role. As the highways are traveled, interesting and attractive byways beckon from all sides. Will they lead too far afield; will they slow down or even stop progress on the main line? He must be careful of:

"Interpreting the simplest symbol wrong,
Missing the gold and treasuring the tin,
Dwelling upon the trivial so long,
And spinning allegory out so thin
That the line parts and neither brawn nor brain
Can splice the mainbrace of the mind again."

He must keep his enthusiasm and not see too many sides of a question, for he might become too cautious. He remembers Thucydides' saying that "Caution is a specious excuse to avoid action"—and action is certainly wanted.

So his colleagues and he consult on the next steps in the program, with the question wide open, "What procedures will yield the most for cancer research?" They have had the problem put to them more than once in this way—"Why don't you folk pick out two or three of the best approaches to cancer and work intensively on them?" They have given the answer more than once, "None of us are wise enough at present to know which approaches are the best, and so we work on many." Years ago Mr. W. H. Donner said he was "playing the field." We may even add the sophistic statement that if we don't find out something about cancer we are bound to learn something about something else, and that might be important too!

In all these highways and byways it helps to consider the changes over the years in the viewpoints about cancer research. One method of arriving at comparisons is to read the programs of the earlier meetings of this Association, and to follow them with the abstracts and then the papers as they appeared. This has been done before, as you know, and several have commented upon the changes. The Cancer Research Association programs years ago took up one day. More time was given to the presentation of individual papers, and the attendance was quite small. In fact, the members of the Association scarcely numbered 200. Today the active membership is 680. Many of the subjects under discussion in our present meetings received consideration then, but the proportions are different. Some subjects in our present program were hardly touched, and some had no representation. Much time was devoted to pathological anatomy, some time to genetics, some to immunology, a trifle to chemistry with, of course, none at all to the use of isotopes. This was before the days of Hevesy and of Schoenheimer's book, "The Dynamic State of Body Constituents." Merely expressing it in another way, most of the presentations seem to be more directly concerned with cancer itself than are many today. I say "*seem* to be more closely connected," for reasons which are quite apparent to this audience. The question is, what was regarded as cancer research then and what is now? Perhaps the easiest way to express what I am thinking is to use one of our own experiences as a text.

When Hammett first came to the Lankenau Hospital Research Institute in 1927 it was after much discussion of how cancer should be approached in the new laboratories which Mr. Rodman Wanamaker of Philadelphia had provided. Hammett asked the question: "What are the chemical differences between cells in mitosis and those in the intervening stages?" The picture in those days was somewhat as follows—here was a fine new building given by a generous donor, in the grounds of the Lankenau Hospital, a general hospital in active work for some seventy years. Naturally, the Trustees of the hospital and the staff were enthusiastic and much interested. It took much explaining when they saw no cancers being investigated but rather normal growing

plants in beakers, normal eggs in incubators, amoebae and various other organisms. We got even with their mild derision sometime later, in a story which most of you know. Now it is common knowledge that cancer is a specialized phase, a deviation from normal growth and development, and must be studied as such.

But more interesting and more challenging to us was the early reaction among our colleagues. I actually had returned a paper on cellular growth in hydra from the editor of a well-known journal of pathology, who stated: "Send it to another journal. This is certainly *not* cancer research!" Just a few years ago at a meeting of this Association a room was filled with attentive listeners to the first eight papers on carcinogenesis—how to produce cancer; whereupon there was a general exodus, and only a few remained to hear the last two papers on attempts to *cure* cancer!

When I am asked, by ever so many people, "Are you making any progress?" my answer is an emphatic "Yes." I quote these experiences and add many others, when I tell of the wide range of subjects treated now. But we must continually reevaluate our programs of research.

The fact that institutions are tax-free, with all that this means, is another origin of responsibility to the citizens at large. How different from a business! This is one of the reasons why quite a few businessmen, newly elected as trustees, rubbing their hands in glee and announcing, "This institution must be operated on a *business* basis." (what they mean is quite variable) find themselves soon confused and confounded with new experiences and problems.

The primary task of many nonprofit corporations is the acquisition of new knowledge—whether it be in science, sociology, or whatever. The sifting of beliefs and the consolidation of older knowledge is another function and, finally, transmission to others. In nearly all there is a pragmatic point of view, or there should be. In cancer research the goal is a better understanding of cancer, which will lead to more certain prevention and better cure. We are, in truth, charged with heavy responsibility, for it is not only of the immediate effects of cancer, for which it is our job to provide better means of control, but we must deal also, directly or indirectly, with the theologic, the sociologic, and the economic aspects.

Gone is the day when cancer could not be discussed, when the very word was taboo with the public. Now cancer is discussed even at dinner tables. Scientists cannot live in "ivory towers" today—if they ever did—especially not those in cancer research. Bacon says, "Those who dwell upon ivory towers have heads of the same material." The public has too much at stake not to be interested in what is going on, and it knows just too much about cancer to be passive. This is very encouraging, for effective action in medical (and other) matters can be taken only when errors in common thinking are corrected and citizens have a basic understanding of the whys and wherefores. This is why typhoid fever, for example, has been almost eradicated. Research workers must assume the obligation, directly or indirectly, of teaching, coaching, and helping their clinical confreres, the teachers at all levels in all schools, the American Cancer Society and other organizations which have the transmission of knowledge as one of their objectives, the press, radio personnel, and others

It is the solid core which must be emphasized and which certainly is not so well known as it might be, rather than the fringe. We may pick out what we think is right and true, i.e., "To teach is to affirm," and later use the other method of setting out ideas for pupils to exercise their own judgment, i.e., "to teach is to put problems"—being careful to keep our own answers from affecting our report of other men's answers.

The ubiquitous question, "What's new?" can have the addition of "and without too much dispute" if it's for general consumption. Apparently the public enjoys the disputes among scientists but, unfortunately, often believes disagreement means that nobody is right. We know that disputes among ourselves are the enzymes that catalyze more thinking and work, and thus are preludes to progress. Isn't that one of the reasons we are here for this meeting? Ideas should emit from us like the sparks from radioactive atoms, for ideas are not mere words but sometimes more important than facts. As far as statements to the public on many controversial points are concerned, perhaps Lord Horder put it very sensibly when he said: "Teach the public a little less than you know." The laboratory worker can perfectly well "let George do it," meaning, let the surgeon, the radiologist, pathologist, and others take the responsibility of the individual cancer patient; but clinicians are not happy with the philosophy of what they do, and too seldom happy in practice. They look to those in the laboratories for better highways and hope that the "tomorrows being born today in laboratories" will be bright and sunny and show clear-cut paths toward better results in patients.

I commented on what some say about too many facts and not enough thinking, and stated that facts should not be neglected. They're not, if

the number of meetings (now amounting to at least two a day throughout the year) are any measure. As a fact, some complain that they have no time to find new facts because of meetings. "Something ought to be done about it" is the wail of most of us. Libraries help accumulate facts and results of thinking too. Journals, pamphlets, brochures, multiply and differentiate faster than our rats, mice, and guinea pigs, and we're afraid to overlook them; so we look over them, concluding that it was a shame to cut down the trees to make the paper on which to print so many words (my speech included).

Not the least of the difficulties is the fashion of meetings to require a paper which must be published. It's bad enough when you know ahead of time, but worse when you've consented to give a talk and then suddenly are confronted with a demand for a formal paper. It calls upon every bit of ingenuity to write the same things in a half-dozen different ways. Speaking extemporaneously is becoming a losing art. It is difficult to do because of time limitations of a minute or two, but a good speaker develops many ideas as he senses the reaction of the audience and responds to it.

There are other areas in which fashion is a dictator. Some years ago a friend showed me a letter from an editor of an important journal, saying that the conclusions in a field fashionable at the time, as submitted in his paper, were at variance with accepted ideas. The author lived to see his conclusions vindicated. It must be interesting to study scientific methods and objectivity as related to scientists as human beings. Some day someone will write a play like Aristophanes' "Clouds," with a little different twist to it.

What I have said, and I could poke more fun at ourselves (including me), does not apply to our Journal *Cancer Research*. It is a good one, and I take pleasure in saying so to our editor and to you, and pay tribute to past editors who carried on so well. There are difficulties in publishing any journal—choosing manuscripts, tactfully rejecting, revising, printing, and so on. It is the financial side I'd like to discuss briefly. The costs of preparation, publication and distribution are far higher than the subscription price. Therefore, our journal must obtain additional funds from advertising or subsidy. Its circulation is too small and too specialized to justify extensive advertising. Therefore, deficits have been liquidated from subsidies which must be obtained every year. We express appreciation to the officers of the Jane Coffin Childs Fund, the Anna Fuller Fund, the Elsa U. Pardee Foundation, the American Cancer Society, and, this year, in addition, to the National Cancer Institute, for the help essential to continuance of our Journal.

In 1935, a group interested in publication gathered at the then Marine Experimental Station of the Lankenau Hospital Research Institute at North Truro, Cape Cod. After discussion for several days another journal was launched—*Growth*—with publication to be financed by payment from research funds of the respective institutions. The idea required cultivation, but the advantages were many: prompt publication, full publication, a low subscription price which covers cost of mailing, and others. The Journal continues now in its seventeenth year, with no advertising, no subsidies, no deficits. Two hundred reprints of each article are furnished to the author, the cost included in the original price per page. From one-quarter to one-half of 1 per cent of the cost of research will pay for publication. Thus, if a piece of research costs $10,000, the price of publication, plus 200 reprints, is about $50. Several other journals in the past few years have used part of this method, so it is not too revolutionary. The experiment has been done with *Growth* and it works—naturally, with certain disadvantages. There need not be relaxation of editorial policy nor penalizing of authors with good papers who cannot pay for them. I recommend consideration of this method for *Cancer Research*, together with the alternative of trying to convince granting agencies, which presumably will remain in action relatively permanently to help research, that publication of results also needs relatively permanent support.

Numerous other highways and byways invite travel, but I'll choose just one or two more for comment. The paths of Chemotherapy have borne increasing traffic during the last few years and much of it has been logically developed and guided. In the theorizing called forth, several principles should be kept in mind. As no two organisms are alike, neither are two cells—a fact that microscopists have noted, particularly pathologists, ever since good lenses were focused on tissues and cells. The differences are reflections of different chemical and physical make-up and activities, and have been actually translated of late into terms of differing chromosome content and various other intracellular mechanisms. It is significant that better results are obtained occasionally when several chemotherapeutic agents are tried. It is also experienced that a patient responding at first to one substance becomes refractory, whereupon another period of improvement ensues upon trial of another material. Chemotherapy combined with irradiation occa-

sionally improves results with a few substances. Mutation and/or selection or multiple damage to different parts?—but this is too big a question for discussion here.

Offhand, it would seem that the efficacy of a chemotherapeutic agent could be appraised in a relatively short time—it causes measurable regression of a tumor or it does not. Unfortunately, it is not that simple, and there is always the matter of controls which are well-nigh impossible in clinical cases. Cancer requires too much prediction with too many variables cluttering the evidence. I so wish for 1,000 patients of the same age, sex, same state of nutrition, same tumor of the same size in the same position—then comparison would be easier; but we will never be provided with this.

Personnel and facilities are limited, and we must choose a few from many applicants for chemotherapeutic trials, with two considerations: one, and foremost, is there a chance of helping the patient? and, two, can we learn something without subjecting the patient to additional discomfort and hazard? Nearly all have advanced malignancy and to choose is not easy. Patients and relatives and friends know it is a last chance, a very long shot indeed, and to be turned away when hopes, however slight, have been rekindled, is to precipitate emotions which perhaps only Dante could express.

Of those whom we have chosen not one, or if the patient does not know the state of affairs (and these are few), not one of the relatives, has failed to agree to the procedures. It goes without saying that full discussion is the prelude to the trial itself. And when failure occurs, as happens too often, it is almost the rule to hear relatives say "I do hope you will be able to do better for the next generation, and hope you have learned something from this patient."

Most cancer research workers not in clinical fields are alert to these dramas and are eager to learn of the special intricacies of human cancer. They realize full well that in the final analysis human beings are the ones we want to help. We are not merely interested in the health and welfare of our mice, rats, Drosophila, frogs, geraniums, and other organisms, except insofar as it is necessary to keep them happy and in good health to act as tools for our work. The members of the staff of our own Institute are polite enough to say they appreciate the discussions I've led for an hour each week, by request, on the subject "Human Tumors." I tell them what we see in human beings, how human beings react, how diagnoses are made, how treatment is determined, how results are appraised. Naturally I dwell on shortcomings, for this is what cancer research should help overcome. Occasionally, not to make it too gruesome, I tell of a triumph or two.

In company with all pathologists I consult with clinical confreres, not only in diagnosis but also in the general care of cancer patients and the possibility of chemotherapeutic trials. I am very grateful to the many physiologists, biologists, chemists, and others who have helped adopt the squalling infant of cancer which Virchow placed upon their doorsteps many years ago. Their unearthing of facts about organisms, tissues, and cells has helped not only in the theory and practice of the pathology of cancer but in its surgery and radiology, and medicine in general. In the thinking about cancer the newly acquired information has used the sharp razor of William of Occam on many older teachings, excellent though they were and useful in their times.

None knows better than the audience here that this address by no means exhausts the possibilities of travel along the highways and byways of cancer research; nor does it even begin to predict what new paths may be opened. It states with conviction, however, that progress is being made, progress in critical experimentation, in evaluation of results, in support, in dissemination of knowledge and methods of practice, in administration.

The responsibility for continued progress is ours; we accept it—in our field and, let us hope in others too, we answer the question "Who is my brother's keeper?"

Nucleic Acids and Tumor Genesis in Broad Bean*

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In an earlier communication, marked changes were reported in the level of nucleic acids during structurally well defined stages in the genesis of crown gall tumors in tomato (9). In view of the distinctive pattern of these changes, it seemed advisable to extend these studies to other plant tumors to ascertain whether nucleic acid changes were, in fact, concurrent with the process of tumorigenesis and to determine whether they were related to the histological and cytochemical changes that occur in tumor genesis. Furthermore, it was important to determine whether these changes were restricted to tumors or were characteristic of plant neoplastic growth in general.

Broad bean (*Vicia faba*, var. English Windsor) was chosen for this study, to compare earlier studies on *Agrobacterium tumefaciens* tumors in tomato with a different host and pathogen. The bean is not susceptible to *A. tumefaciens*, but when inoculated with *A. rubi* does form tumors which are grossly distinct from those formed by tomato and many other hosts (4). Furthermore, bean tissues possess large cells and nuclei which can be studied histochemically.

MATERIALS AND METHODS

Bean seeds were treated with 0.1 per cent Semesan, soaked overnight, and planted in loam soil. The pots, each containing three plants of uniform size, were placed at random on a greenhouse bench. Normal photoperiod was supplemented with 350–400 foot candles of light (pot level), supplied by 200-watt incandescent bulbs. Twenty-one days after planting, the fifth internode was inoculated with *Agrobacterium rubi* (Hildebrand) Starr and Weiss (obtained from Dr. L. C. Coleman), by a single puncture with a trident previously dipped into the culture; control plants were punctured with a sterile trident. Stem segments were collected at various time intervals after treatment and sliced into thin discs about 0.3 mm. thick. All collections were made at noon on the appropriate day, the first 1 hour after treatment. Slices included only the puncture and were put in water, blotted, and weighed before chemical examination. For cytological examination, slices were immediately put into 3:1 absolute alcohol: acetic acid, or into 10 per cent neutral formalin for fixation. All dry weight values were obtained after 24 hours at 95° C.

Desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) were estimated by the method of Ogur and Rosen (13), with 200–300 mg. fresh weight of tissues for each analysis. The DNAP extraction temperature was reduced to 70° C.; the number of cold and hot extractions was increased by one each. Percentage standard errors of aliquot portions of a homogenate of *Vicia faba* stems were 3 per cent for RNAP and 2.5 per cent for DNAP. Percentage standard errors for sextuplicate analyses of individually weighed samples were 9 per cent for RNAP and 4 per cent for DNAP. In these studies, three weighed samples were analyzed.

For the study of auxin-induced neoplasms of bean, the same test procedures were followed. Three per cent indoleacetic acid (IAA) in lanolin (12) was applied in a 1-cm. ring to the middle of the fifth internode, making available a large presentation area for penetration of the auxin (10). Control plants were not treated; lanolin alone had no effect. The lanolin paste was removed prior to slicing. Samples were taken only from the treated areas.

Fixed tissues were prepared in the usual manner for histological study. Relative amounts of DNA in individual nuclei were estimated by microphotometric determinations of Feulgen-stained sections (DNA-Feulgen), as previously described (17). Sections of both alcohol-acetic and formalin-fixed tissues were stained for 1 hour with Feulgen reagent after hydrolysis for 12 minutes at 60° C. in 1 N HCl. Longitudinal sections were found most satisfactory to assure measurement only of whole, spherical nuclei. Because bean nuclei were too dark to measure accurately at 560 mμ (absorption peak of the Feulgen-DNA complex), measurements were made off the peak at 600 mμ.

RESULTS

Biochemical.—The DNAP of prospective bean tumor tissues rose sharply following inoculation, reaching a peak at 2 days (Chart 1). Subsequently, there was a decrease in the DNAP level to a point below that found at the time of inoculation. The lowest level of DNAP in tumorous tissue was reached at 5 days, followed by a secondary rise to a plateau from 9 days until the close of the study. In control material, a steadily decreasing level of DNAP was observed. That this peak in DNAP is not due to an increment of bacterial DNA inci-

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dent to multiplication of the bacteria in the tissues was determined by inoculating susceptible plants with a completely avirulent strain of *Agrobacterium*.[1] Although these bacteria multiplied normally in the inoculated tissues (8), the DNAP curve for the critical period was identical with that of punctured control tissues.

Changes in RNAP were less striking (Chart 2). Immediately following inoculation, there was a small, possibly significant, rise in RNAP. During the next 4 days, RNAP in control and inoculated tissues showed a parallel decrease, followed by a secondary rise from the 9- to 20-day period. Except for the small initial rise in RNAP of prospective tumorous tissues at the 1- to 2-day period, no significant differences between control and tumorous tissues were noted.

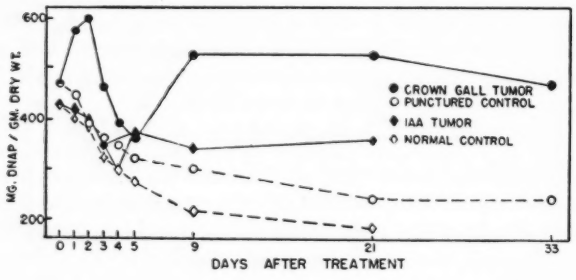

CHART 1.—Levels of desoxyribonucleic acid phosphorus during the genesis of tumors and auxin-induced neoplasms of broad bean.

No differences were found in DNAP levels of either auxin-treated plants or untreated controls during the first 4 days (Chart 1). When the auxin-neoplasm became macroscopically evident (5 days after IAA treatment), there was a secondary rise in DNAP to a level slightly below that found at zero time. This level remained constant throughout the period of study. RNAP levels of IAA-treated tissues were strikingly similar to those DNAP changes observed for the early period of crown gall development (Chart 2). There was an initial rise, reaching a peak 2 days after application, followed by a fall to the level of untreated control tissues within 9 days. No further changes were noted.

Histological.—Initiation of tumor genesis was histologically detected within 2 days after inoculation by increased cambial activity, and within 3 days by enlargement of cortical, endodermal, and vascular parenchyma cells. Tumorous tissues showed extensive cell division within 5 days (Fig. 2) in comparison to control stems (Fig. 1). Complete disorganization of normal stem architecture occurred within 20 days following rapid proliferation of all parenchymatous tissue elements as well as cell enlargement, de-differentiation, and division of perimedullary tissue, pericyclic fibers, and cortical fiber bundle cells.

The high degree of normal stem architecture maintained in IAA-induced proliferations, as reported by Palser (14), is in striking contrast to the disorganization found in the autonomous growth of bean tumors. Cell enlargement and initiation of cell division were reported by Palser to occur within 30 hours, while divisions in most parenchymatous tissues were found at 48 hours after treatment of decapitated bean stems.

Cytochemical and cytological.—The amount of DNA-Feulgen complex per nucleus was determined in over 800 nuclei of tumor and control tissues. Values from normal tissues were those expected from previous findings (17, 18). Cambial telophase nuclei contained the diploid or 2C amount of DNA and prophases, twice the diploid or 4C amount (dotted lines of Chart 3); interphase values spread between these limits. It thus appears that DNA synthesis here occurs in interphase, as found in many other plant and animal tissues (see 18 for review). In spite of the marked DNAP increase shown biochemically in 2-day tumor tissues, no significant differences in DNA-Feulgen values over the control were found. It is unlikely that, if such an increase included Feulgen-positive material, it would be unnoticed in measurements on individual nuclei. The increase apparently cannot be associated primarily with cell division, since tumor proliferation did not reach a maximum until 5 days or later after inoculation. By this time, the DNA found biochemically dropped sharply. Also, at the time of the DNA decline there was no evidence of cell death, except in the wound pseudocicatrice. This degeneration was essentially the same in treated and control sections (Figs. 1 and 2). No Feulgen-positive material was seen in the cytoplasm of control or inoculated tissues.

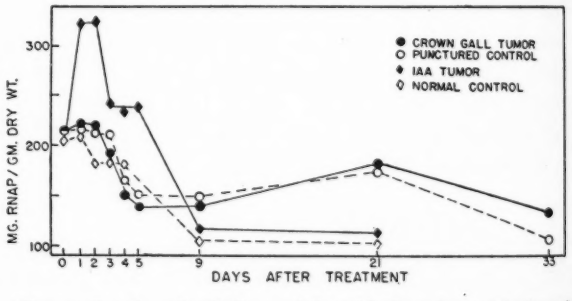

CHART 2.—Levels of ribonucleic acid phosphorus during the genesis of tumors and auxin-induced neoplasms of broad bean.

[1] (Klein, unpublished data, 1953).

Determinations of DNA-Feulgen per nucleus in mature tumorous tissue 33 days after inoculation, and in wound periderm of 33-day-old control tissues, gave values grouping well into polyploid classes: 2C, 4C, 8C, and 16C. Division of polyploid nuclei occurred in the maturing tumor 20 and 33 days after inoculation. No polyploid divisions were found in wound periderm of 20- and 33-day control stems. Details of the histology and nucleic acid changes accompanying tumor development will be published elsewhere.

Contrary to previous reports of aberrant nuclear division as characteristic of tumor tissues of plants (16, 19), only two instances of micronuclei adjacent to reconstruction nuclei, and only one case of a bi-nucleate cell were observed. Similar abnormalities may be seen in normal tissues. As suggested by Levine (11) aberrant divisions, therefore, are not necessarily characteristic of plant tumor cells.

DISCUSSION

Several critical points in the relation of nucleic acids to tumor genesis have been examined. Primary among these was the demonstration that an increase in DNA 1–2 days after inoculation of virulent bacteria occurred in the genesis of crown gall tumors (9) but not in auxin-induced neoplasms.

The distinctive pattern of DNAP changes in tumor genesis, as determined biochemically, shows little direct correlation at present with either histological or cytochemical observations. Satisfactory technics, as have been applied in the case of animal tissue studies (15), do not exist for direct cell number estimation in plant tissues. Total nitrogen as a base for cell number has also been shown to be unsatisfactory (9, 12). Consequently, no exact correlations between biochemically observed levels of DNAP and relative amounts of DNA-Feulgen per nucleus have been attempted here. Since the 1- to 2-day peak in DNAP was not reflected in Feulgen determinations, this increase seems best interpreted as either a result of specific action of prospective tumor cells, or a product of virulent, tumor-inducing bacteria. Avirulent bacteria which do not induce tumor formation nor evoke the DNAP peak[1] can be altered into tumor-inducing forms which do evoke this peak by treatment with the nucleic acid of virulent forms. Strains and species of Agrobacterium, including *A. rubi*, possessing one host range, can acquire new host ranges by these technics (8). It may be concluded that the DNA peak represents synthesis of a specific nucleic acid by virulent bacteria, this nucleic acid apparently playing an etiological role in the transformation of normal cells to tumor cells. In later stages of tumor growth, elevated DNAP levels may be due to larger numbers of cells per unit weight of tissue, and to the increased proportion of polyploid cells in the crown-gall tissue mass (Rasch, unpublished data, 1952).

In contrast to the unusual nucleic acid pattern during the early phase of tumor genesis, DNAP and RNAP levels during the development of auxin-induced proliferations present no findings which differ from those observed in studies on normal cell growth. Heightened RNAP levels found 2 days after application of IAA may be correlated with histological findings of enlargement of affected cells at that time (14) and possibly with protein synthesis. High rates of protein synthesis have been found to accompany auxin-induced cell enlargement in pea stems by Christiansen and Thimann (3). Stimulation of mitosis in auxin-induced neoplasms during the initial response to treatment (14) was reflected biochemically by a steady decline in DNAP levels during the critical 1- to 3-day period. Although timing of histological responses to treatment in auxin neoplasms may be more rapid than that found here for the tumors, changes of DNAP levels in the two cases cannot be directly related to cell numbers or cell division.

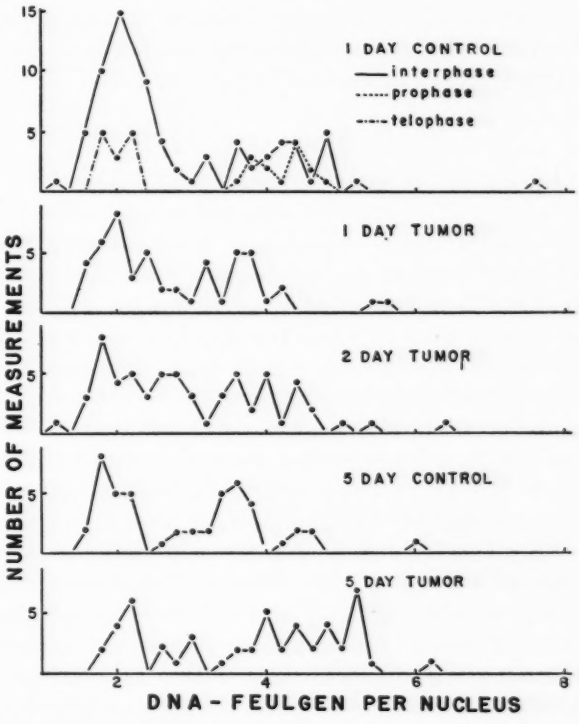

CHART 3.—Amounts of DNA-Feulgen, in arbitrary units, in individual nuclei from control and tumorous stem tissues of broad bean.

This evidence would further indicate that the DNAP peak of the prospective tumorous tissues is not characteristic of normal growth responses.

From Coleman's previous study on mitotic figures from IAA-induced divisions of cortical parenchyma (5), and the present cytochemical study of interphase nuclei of several stem tissues, it can be concluded that polyploidy alone is not sufficient to characterize tumor tissue. Similar findings for several mammalian tumors have been recently presented (1). Divisions of highly polyploid nuclei, however, may be characteristic of tumor development, for only diploid division figures were found in the older control tissues after completion of the mitotic response to wounding. Polyploid divisions, moreover, are known to be extremely rare in normal tissues (6, 7).

SUMMARY

Levels of desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) were determined at various times during the genesis of tumors and auxin-induced proliferation on stem tissues of broad bean. Prospective tumorous tissues showed a sharp peak of DNAP levels 2 days after inoculation, a maximum depression at 5 days, and a gradual increase to a plateau by 9 days. No marked changes in RNAP were noted. Auxin-induced neoplasms showed a sharp initial rise in RNAP at 2 days after application of IAA, but only slightly elevated DNAP levels.

Initiation of tumor genesis was histologically detected within 2 days after inoculation by increased cambial activity and at 3 days by enlargement of stelar parenchyma. Tumorous tissues showed extensive cell division within 5 days. Complete disorientation of normal stem architecture occurred within 20 days.

Photometric determinations of over 800 Feulgen-stained nuclei of inoculated and control tissues showed no significant increase in relative amounts of DNA per nucleus in tumor tissues during the first 5 days. No evidence for cytoplasmic localization of DNA was found. Although the number of and division of polyploid nuclei may be characteristic of tumorous growth, both wounded and inoculated tissues showed a pattern of DNA values essentially similar to that found in areas of meristematic growth, as compared to mature, nondividing tissues.

Comparisons of histological and cytological findings with biochemically observed changes in nucleic acid levels accompanying crown gall tumor genesis have indicated that the 1- to 2-day peak of DNAP may be a result of specific action of prospective tumor cells, or a product of virulent tumor-inducing bacteria.

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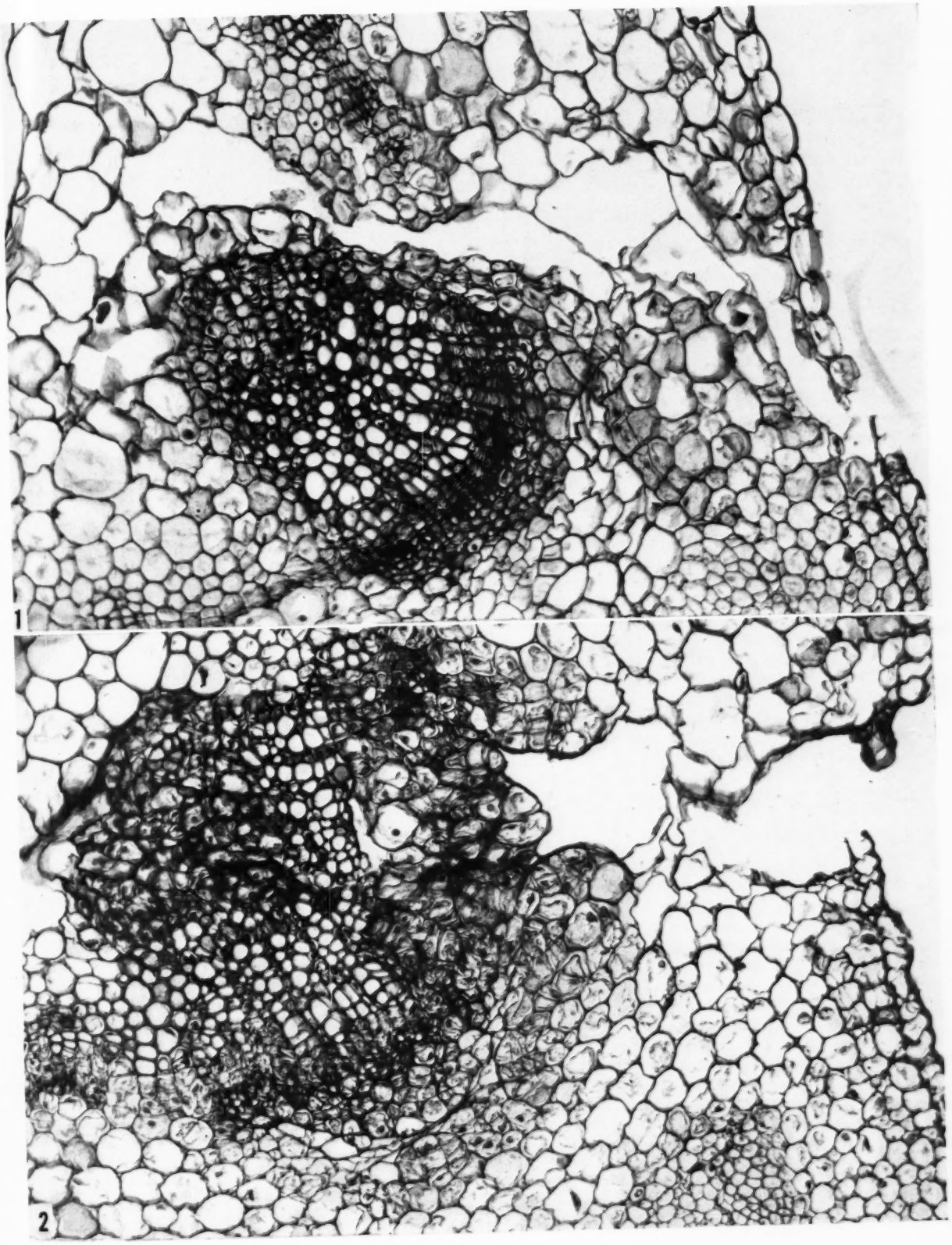
Figs. 1 and 2.—Broad bean stem x-sections. Fig. 1.—control 5 days after wounding; Fig. 2.—tumor 5 days after inoculation. ×110.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Effect of Dose and Hormones on Tumor Production in Rats Given Emulsified 9,10-Dimethyl-1,2-benzanthracene Intravenously*

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Intravenously administered oil emulsions which contain 9,10-dimethyl-1,2-benzanthracene have been shown previously to produce a fairly high incidence of mammary tumors in female rats (5). Male rats, however, developed few tumors, and these were chiefly papillomas arising from the sebaceous gland ducts of external ears. During these and subsequent studies it became apparent that tumor incidence and dose of carcinogen were closely related, and that, at doses of 4 times the maximum previously employed, an incidence of approximately 90 per cent was achieved in 19 weeks.

The simultaneous injection of hormones such as α-estradiol was also investigated because of (*a*) the difference noted earlier between the sexes, (*b*) the possibility that the endogenous supply of such hormones was not constant between female rats nor in the same female rat, and (*c*) the effect of such hormones on the mammary gland. The present paper deals with some of these experiments.

EXPERIMENTAL

Emulsions of oil containing 9,10-dimethyl-1,2-benzanthracene (DMBA) were prepared by the blending technic previously described (5) and had the following composition:

Ingredient	Mg/ml
Corn oil[1]	50
Phosphatide[2]	10
Demal-14[3]	10
Triton-WR-1339[3]	10
DMBA[4]	0.44
α-estradiol (when present)[5]	0.005– 0.05
Diethylstilbestrol (when present)[5]	0.005– 0.05
Dextrose (5 per cent in water) to make	1 ml.

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The emulsion was autoclaved in nitrogen-flooded sealed glass ampules at 15 pounds/square inch for 15 minutes and stored in the dark. Such preparations remained stable for months.

Female rats, descendants of the Sprague-Dawley strain,[6] weighing 150–200 gm. were used throughout and were fed a commercial stock ration[7] and water ad libitum. The emulsions were injected through a tail vein, while the animals were under light ether anesthesia. After receiving all their respective injections, the animals were palpated weekly to determine the appearance of both the first and subsequent tumors. Tumor-bearing animals were sacrificed when the neoplasms seriously interfered with survival. All tumors and the liver, heart, lungs, spleen, kidneys, and adrenal glands were placed in 10 per cent formalin fixative, and histological sections were prepared and stained with hematoxylin-eosin.

In previous experiments (5), the maximum total dose of DMBA given was approximately 4.3 mg/100 gm of body weight, and this was given over a period of 13 consecutive weeks. A similar dose was used in more recent experiments (Group 1) but was given according to the following injection sequence: three injections spaced 1 day apart followed 8 weeks later by a similar set of injections. Each injection of the DMBA emulsion amounted to 0.5 ml/100 gm of body weight.

[1] Mazola; generously supplied by Corn Products Refining Company, Argo, Ill.

[2] Phosphatides prepared as follows have proved as satisfactory as the fraction used in earlier studies (6):

Four hundred gm. of grade RG lecithin generously supplied by the Glidden Company, Chicago, Ill., was dissolved in 1.6 liters of redistilled petroleum ether and filtered through an asbestos-type bacterial retaining filter. The filtrate was slowly poured into 4 liters of redistilled acetone with vigorous stirring, and the precipitate was collected by filtration. The precipitate was redissolved in petroleum ether and reprecipitated from acetone as before. The product was washed well with acetone, and the residual solvent was removed by means of a dry stream of nitrogen. The phosphatides were stored in a tightly capped bottle and kept under refrigeration.

[3] The Demal-14 was supplied by the Emulsol Corporation, Chicago, Ill., and the Triton WR-1339 by the Rohm and Haas Company, Philadelphia, Pa.

[4] Eastman Kodak Company, Rochester, N.Y.

[5] Generously supplied by Dr. Hailman of the Upjohn Company, Kalamazoo, Mich.

[6] Obtained from the Charles River Breeding Laboratories, North Wilmington, Mass.

[7] Purina Laboratory Chow. Ralston Company, St. Louis, Mo.

Other groups of rats received similar injections of emulsion which furnished in addition to the DMBA a total of either 0.015 or 0.15 mg. of *a*-estradiol (Groups 2, 3) or diethylstilbestrol (Groups 4, 5)/100 gm of body weight. The injection sequence for animals which received the high amount of estradiol (Group 3) was as follows: two injections spaced 1 day apart, followed 2 months later by four injections spaced 1 day apart. The numbers of animals in Groups 1, 2, 3, 4, and 5 were 22, 22, 25, 23, and 22, respectively. The results of these experiments are given in Charts 1 and 3 and Table 1.

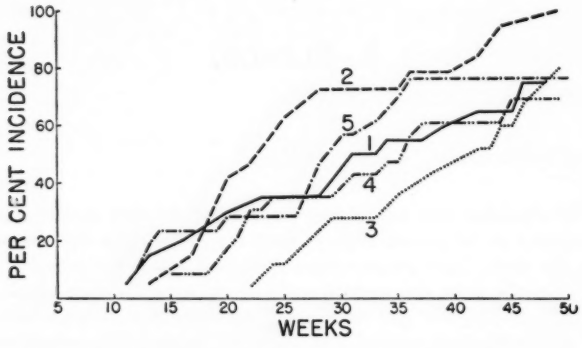


CHART 1.—Incidence of tumors in rats which received 1.32 mg dimethylbenzanthracene intravenously/100 gm body weight. In addition to the DMBA, the animals also received the following intravenous supplements: Group 1, nothing; Groups 2 and 3, 0.015 and 0.15 mg. of *a*-estradiol, respectively; and Groups 4 and 5, 0.015 and 0.15 mg. of diethylstilbestrol, respectively. See text for comment on Group 3.

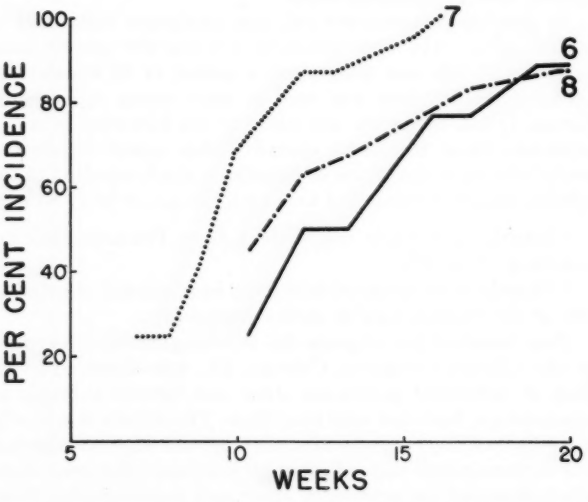
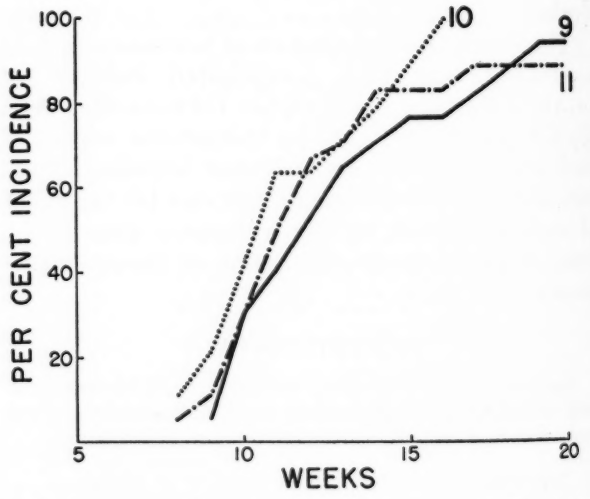


CHART 2, *A*.—Incidence of tumors in rats which received 5.28 mg DMBA intravenously/100 gm body weight. In addition to the DMBA, the animals also received the following intravenous supplements: Group 6, nothing; Group 7, 0.60 mg. *a*-estradiol; and Group 8, 0.60 mg. diethylstilbestrol.



B. Same protocol as given in Part *A*. Groups 9, 10, and 11 correspond to Groups 6, 7, and 8, respectively.

An experiment was undertaken in which a total dose of 5.28 mg. of DMBA/100 gm body weight was administered according to the previous injection schedule. In addition to the DMBA control group (Group 6), other groups of animals received simultaneously in the emulsion 0.6 mg. of either *a*-estradiol (Group 7) or diethylstilbestrol (Group 8)/100 gm of body weight. These groups were subsequently duplicated as Groups 9, 10, and 11. Other groups of rats received emulsion which contained either estradiol (Group 12) or diethylstilbestrol (Group 13) but no DMBA. The animals in Group 14 received six injections of 2 ml/100 gm body weight of an emulsion which contained all the ingredients except the DMBA and hormones. Those in Group 15 served as uninjected controls. Weekly palpation of these animals was begun after the last injection. Animals were sacrificed only when in moribund condition, and sections of the tissues and tumors were taken for histological examination as in the other experiments. The numbers of animals in Groups 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 were 28, 24, 24, 17, 19, 18, 14, 21, 42, and 30, respectively. The results of these studies are given in Table 1, Charts 2*A*, 2*B*, and 3.

RESULTS AND DISCUSSION

Rats which received 1.32 mg. of 9,10-dimethyl-1,2-benzanthracene/100 gm body weight developed mammary tumors at a fairly slow rate, as seen from the data given in Chart 1. In 48 weeks only 75 per cent of the animals which received DMBA alone had tumors, in agreement with results published earlier (5). In the animals which received 0.015 mg of *a*-estradiol/100 gm body weight in addition to the 1.32 mg. of carcinogen (Group 2), tumors appeared earlier than in the control rats (Group 1), and the tumor incidence and average number of tumors per rat were higher. Unfortunately, the rats in Group 3 which received 0.15 mg of *a*-estradiol/100 gm body weight cannot be directly compared, because the injection sequence was unavoidably different; however, here too, the influence of the hormone was apparent. Rats which received diethylstilbestrol and 1.32 mg DMBA/100 gm body weight had an elevated incidence rate only when given 0.15 mg. of hormone (Group 5) but had a greater average number

of tumors per rat with either the 0.015- or 0.15-mg. dose of hormone. An interesting difference between the effects of estradiol and diethylstilbestrol is that administration of the latter compound did not increase the total number of rats that developed tumors, whereas estradiol did. Others have reported experiments in which rats were given methylcholanthrene by gastric instillation (8), and estradiol was found to decrease the latent period for mammary tumor production, increase the incidence, and cause a more glandular type of tumor to develop.

As has been found in other studies (1–3), the dose of carcinogen was an important factor in the present investigations. This effect is evident by comparison of Groups 1 and 6. As shown in Charts 2*A* and 2*B*, 90 and 94 per cent of the animals in Groups 6 and 9 which received approximately 5.28 mg of DMBA/100 gm body weight developed one or more tumors within 19 weeks. The tumors were mainly mammary adenocarcinomas in contrast to the fairly large number of mammary adenofibromas, adenomas, and fibromas, and ear carcinomas found in the earlier studies (5).

As shown by the data presented in Charts 2*A*, 2*B*, and 3 (Groups 7 and 10), α-estradiol injected simultaneously with the higher dosage of carcinogen caused tumors to appear earlier, in 100 per cent of the animals, and approximately twice as many to develop as in the control DMBA group. At 10 weeks in Group 7, about 70 per cent of the animals each had an average of 2.5 tumors, and at 16 weeks all animals had tumors with an average of 4.8 tumors/rat (Chart 3). Almost without exception the tumors in this group were mammary adenocarcinomas (Table 1).

The diethylstilbestrol with high doses of DMBA likewise caused a more rapid production of tumors and a greater average number of tumors per rat, as compared to the controls, but the effect was not quite so great as that of α-estradiol.

Neither estradiol nor diethylstilbestrol alone in an amount 5–7 times that used with DMBA has produced tumors even after 25 weeks (Groups 12 and 13). These compounds have been shown to cause tumors to develop in rats (4, 7), but it seems likely that much greater doses would have to be used intravenously to produce tumors in the absence of DMBA. In the present experiments the dose of hormone used in conjunction with the DMBA was chosen because that dose of hormone alone did not produce tumors, yet it did enhance tumor production with DMBA. Animals which received emulsion which contained neither DMBA nor hormones (Group 14) and those which were not injected at all (Group 15) developed no tumors.

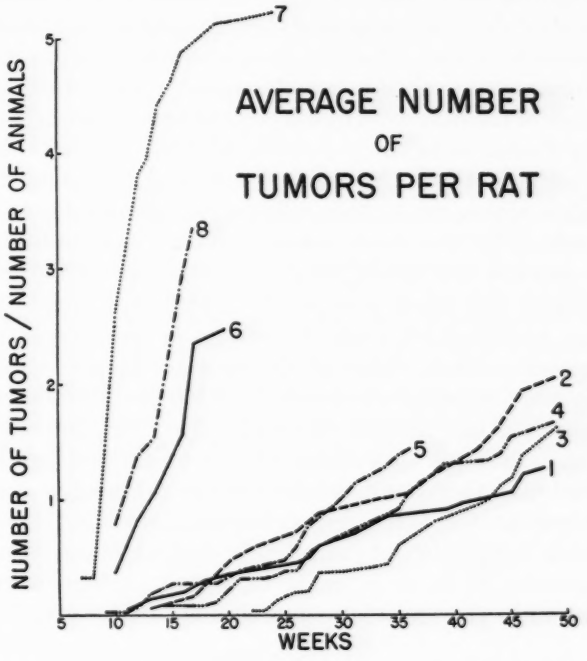


CHART 3.—Average number of tumors per rat for all of the animals in the groups shown in Charts 1 and 2*A*.

TABLE 1

MICROSCOPIC DIAGNOSIS OF TUMORS

	GROUP					
	1	2	3	4	5	7
Duration of experiment (weeks)	52	52	52	52	52	16
Per cent of rats with tumors	75	100	80	80	76	100
No. of animals examined microscopically	21	19	24	19	20	24
Adenocarcinoma of breast	12(8)*	20(11)	22(12)	21(12)	16(9)	114(24)
Adenoma of breast	5(3)	6(5)	0	5(4)	3(3)	1(1)
Fibroadenoma of breast	3(2)	5(2)	8(6)	8(4)	2(2)	0
Fibroma of breast	0	0	4(2)	0	1	0
Fibrosarcoma, dermal	0	0	0	2(2)	0	0
Keratinizing squamous-cell carcinoma of sebaceous gland of external ear	4(4)	2(2)	5(5)	1(1)	5(4)	0
Keratinizing squamous-cell papilloma of sebaceous gland of external ear	0	0	0	0	1	0

* Larger figure indicates total number of tumors, figure in parentheses refers to number of animals with tumors.

Tumor production by means of the method employed in the present paper offers the following advantages in the study of mammary cancer: (*a*) Rapid and reproducible tumor production, (*b*) tumor incidences of 100 per cent, (*c*) range in number of tumors per rat, (*d*) homogeneity of the type of tumors produced, and (*e*) a means for the simultaneous administration of other lipid-soluble materials. The latter has the advantage that each particle of such a fat emulsion is in itself a separate nonaqueous solution which carries with it some of each of the dissolved materials and thus, if absorbed by tissue cells, may furnish all the lipid-soluble components of the emulsion at a given instant. This circumvents the reliance on chance that is ordinarily involved when various lipid-soluble materials are given singly or together by other means and fairly comparable rates of absorption are assumed for each. It should be pointed out, however, that, although the primary target in the present experiments proved to be the mammary gland, other tissues, especially the liver, initially take up large quantities of the emulsified lipids and may then act as sources of the carcinogen and hormone for later redistribution, provided that such tissues do not completely destroy these compounds. It is of interest that few tumors were found in tissues other than the mammary gland.

SUMMARY

Tumor production in female rats by means of intravenously administered 9,10-dimethyl-1,2-benzanthracene (DMBA) has been further studied from the standpoint of the dose of carcinogen given and the influence of simultaneously administered α-estradiol or diethylstilbestrol. Administration of approximately 5.2 mg of DMBA/100 gm body weight produced a tumor incidence of approximately 90 per cent in 19 weeks, in contrast to an incidence of 80 per cent in 47 weeks with a dose of 1.25 mg. The rats which received the higher dose of carcinogen had twice as many tumors per rat as the others.

The simultaneous administration of 0.6 mg. of estradiol caused an earlier appearance of tumors, increased the tumor incidence to 100 per cent in about 16 weeks, increased the number of tumors per rat, and yielded mammary adenocarcinomas almost exclusively. Six-tenths milligram of diethylstilbestrol was almost as effective as estradiol. Neither hormone alone in doses up to 4 mg/100 gm body weight produced any tumors.

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Effect of Ethionine on Tumor Growth and Liver Amino Acids in Rats*

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It was anticipated from earlier studies on growth retardation of rats (8) that ethionine might be somewhat specific for rapidly growing tumors. Since ethionine interferes with protein synthesis, possibly through its role as a competitive antagonist of methionine, it was assumed that its effect would be most marked in tissues with a low concentration of endogenous methionine. It has been reported in previous studies from this laboratory (2) that the methionine content of the U.C.L.A. fibrosarcoma was lower than that of normal rat connective tissue. The experiments described here were concerned with the growth of tumors and the levels of certain total and "free" amino acids in the livers of normal and tumor-bearing rats with and without the administration of ethionine.

EXPERIMENTAL

DL-Ethionine (U.S. Industrial Chemical Co.) was either incorporated into the basal ration (see Table 1) or injected subcutaneously into the rats (Long-Evans strain) as a physiological saline solution containing 20 mg of DL-ethionine/ml. Each animal in a given experiment received subcutaneous inoculations of either the U.C.L.A. fibrosarcoma or the Jensen sarcoma at one inguinal ventral site by trocar implantation of a cube of tissue approximately 75 c. mm. in volume taken from the same tumor. After the tumors became palpable, their areas were estimated from caliper measurements. The animals were divided into groups of individuals of approximately the same body weight and with tumors of approximately the same area. Possible systemic effects of DL-ethionine on nontumor-bearing rats were investigated in some experiments. The pertinent data are summarized in Table 2. In all experiments tumor growth was followed by means of caliper measurements, body weights were determined periodically, and food consumption was recorded (but omitted to conserve space) in the indicated experiments. In Experiment VII (see Table 2) the control rats were housed in individual cages and were restricted to the average food intake of the experimental animals.

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At the termination of Experiments V, VI, and VII, the tumors and livers were rapidly excised, weighed, frozen with solid carbon dioxide, and stored at about −20° C. The livers of animals from Experiment V, pooled in groups as shown in Table 3, were homogenized in a Waring Blendor. Aliquots were taken for analysis of total nitrogen by Kjeldahl, total fat by the method of Jensen *et al.*, (3) and arginine, glycine, methionine, and threonine by the microbiological assay procedures of Dunn *et al.* (1). For the determination of nonprotein liver amino acids, the protein was precipitated with tungstic acid by the method of Schurr *et al.* (7), the filtrate was reduced to a syrup by evaporation *in vacuo* at 60°C., and the syrup was refluxed for 24 hours with 6 N HCl to hydrolyze any peptides present.

TABLE 1

COMPOSITION OF BASAL RATION

Constituent	Per cent
Casein, vitamin-free (Nutritional Biochemicals)	22
Sucrose	67
Salt mixture (Sure's No. 1)	4.5
Corn oil (Mazola)	6.0
Cystine	0.3
	mg/kg
Thiamine chloride	5
Pyridoxine	5
Riboflavin	10
Niacin	100
Calcium DL-pantothenate	100
p-Aminobenzoic acid	150
Inositol	400
Choline hydrochloride	1,000
Biotin	0.2
Folic acid	0.2
Vitamin B_{12}	0.15
Vitamin E	50
Vitamin K	1
	units/kg
Vitamin A	20,000
Vitamin D	2,000

RESULTS AND DISCUSSION

The effect of DL-ethionine on the body weight of nontumorous rats is shown in Table 2. When fed at a level of 0.5 per cent, there was an early marked loss in weight followed by stabilization at a lower weight. At the lower injected dosages there was a tendency to regain weight after the initial loss. At very high dosages the weight loss

TABLE 2

Data on Experimental Animals and Tumors*

Exp. No.	Tumor	No. rats	Sex	Ethionine	Initial tumor Age (days)	Initial tumor Size (mm^2.)	Final tumor Age (days)	Final tumor Size (mm^2.)	Final tumor Weight (gm.)	Final body wt.	Wt. change	Liver wt. (wet)
	fibrosarc.	9	F.	none	8	132	43	none		205	+44	
	"	6	"	none		186	43	2,100		171	+19	
	"	1	"	none		56	55	2,232		230	+40	
I	"	17	"	0.5 per cent		139	43	none		168	+ 6	
	"	1	"	0.5 per cent		144	43	1,833		130	−10	
	none	9	"	none						209	+39	
	none	9	"	0.5 per cent						156	−15	
	fibrosarc.	10	"	none	4	58	34	none				
II	"	2	"	none		50		1,634; 240				
	"	7	"	1.0 per cent		60		none				
	"	7	"	1.0 per cent		80		1,396				
	fibrosarc.	6	"	none	12	143	35	none				
III†	"	2	"	none		183		933				
	"	4	"	0.5 per cent		150		none				
	"	4	"	0.5 per cent		171		1,155				
IV	Jensen	21	"	none	5	150	19	2,050		241	+ 1	
	"	24	"	1.0 per cent		150		1,530		194	−52	
	Jensen (small)	7	M.	none	5	83	26	282	2.1	336	+ 1	12.4
	Jensen (large)	17	"	none		150		2,400	46.9	313	−16	13.9
	Jensen (small)	16	"	0.25 per cent		125		145	1.3	308	−15	13.9
	Jensen (large)	10	"	0.25 per cent		125		1,680	28.2	300	−33	13.3
V	Jensen (small)	18	"	0.50 per cent		125		210	1.4	276	−46	13.0
	Jensen (large)	4	"	0.50 per cent		135		1,750	31.6	300	−31	15.2
	none	15	"	none						330	+ 9	13.6
	none	15	"	0.25 per cent						333	− 9	15.7
	none	14	"	0.50 per cent						320	−18	15.4
VI	Jensen	22	"	none	4	150	19	1,200	20.6	238	−68	10.1
	Jensen	22	"	20 mg.‡		155		975	20.9	222	−42	8.8
	Jensen	25	F.	none	7	300	16	1,400	26.9	229	−12	11.4
VII	Jensen	43	"	§		300		750	7.2	191	−51	7.9
	none	10	"	none						236	− 8	8.8
	none	15	"	§						198	−53	7.7

* The tumors which are included in each subgroup all showed the same type of growth curve.

† Not included are thirteen animals whose tumors regressed before the 18th day and were not treated.

‡ 20 mg/rat/day injected subcutaneously.

§ 200 mg. ethionine injected subcutaneously on the 7th and 8th days; 100 mg. ethionine injected daily from the 12th through the 15th day.

TABLE 3

Total Amino Acids in Livers of Control and Tumor-bearing Rats

Experiment V			Amino acid (per cent in protein calc. to 16 per cent N)				
Tumor	No. Rats	Ethionine	Arginine	Glycine	Serine*	Methionine	Threonine
Jensen (small)	7	none	5.43	4.97	6.28	2.15† 2.48	4.40
Jensen (large)	17	none	5.44	4.88	6.48	2.09 2.38	4.31
Jensen (small)	16	0.25 per cent	5.08	4.77	6.24	2.07 2.27	4.52
Jensen (large)	10	0.25 per cent	5.48	4.74	6.37	2.13 2.48	4.39
Jensen (small)	18	0.50 per cent	5.25	4.74	6.37	2.11 2.40	4.54
Jensen (large)	4	0.50 per cent	5.43	4.80	6.54	2.07 2.51	4.54
None	15	none	5.34	4.64	6.65	2.18 2.58	4.69
None	15	0.25 per cent	5.28	4.88	6.41	2.12 2.40	4.70
None	14	0.50 per cent	5.42	4.99	6.60	2.14 2.54	4.83

* Serine was determined by Camien and Dunn's improved unpublished method.

† This value and the one listed first in each group was obtained with *Leuconostoc mesenteroides* P-60 which responds only to L-methionine (unpublished data from the authors' laboratory). The value listed second in each case was obtained with *Lactobacillus fermenti* (1) which responds to both L- and D-methionine. The higher values found with *L. fermenti* may indicate slight racemization during hydrolysis of the livers.

was continuous. The development of resistance may be explained by a detoxification mechanism or, possibly, by competitive antagonism of 'free' methionine which increased (see Table 4) in the livers of ethionine-treated rats. As would be expected on this basis, longer times were required to overcome the toxic effects of larger doses of ethionine. In nontumorous groups deaths occurred only in rats receiving two injections of 200-mg. each of DL-ethionine on consecutive days. Treated animals, bearing large tumors which did not regress, tended to die sooner than the controls. The weight changes were similar for nontumor-bearing animals and those with tumors which regressed or grew slowly.

The administration of ethionine tended to reduce food intake as observed in Experiment V where the average daily intake was 8.8 gm/day for control animals as compared to 7.4 gm/day for tumor-bearing animals fed 0.25 per cent ethionine and 6.6 gm/day for tumor-bearing animals fed 0.50 per cent ethionine. On the other hand in Experiment I there was no difference in food intake of the control and experimental groups, while the effect of ethionine was similar to that noted in Experiment V. In Experiment VII, in which the control rats were limited to the average food intake of the treated animals, the tumors grew very rapidly. The report of Sugiura and Stock (9) that there was little or no inhibition of tumors in mice maintained for 9–14 days on one-third to one-fourth of the normal dietary intake has been confirmed by the authors' experiments in which it was shown that the growth of established Jensen sarcoma (7-day implants, area 300 mm.^{2}) was not altered significantly during 2 weeks of starvation. It appears, therefore, that neither the weight losses nor the effect on tumor growth induced by ethionine depended primarily on differences in food intake.

The administration of ethionine under the conditions of Experiment I resulted in an early retardation of growth and an increase in the number of regressions of the U.C.L.A. fibrosarcoma (Chart 1). The tumors of nine of the sixteen control rats and seventeen of the eighteen experimental animals regressed completely. In one control animal it was observed that, although there was no appreciable growth of tumor until the 32d day after implantation, the tumor grew rapidly thereafter and eventually caused the death of the animal. There is, therefore, a fine balance between factors operating to influence establishment and regression of some tumors. It is of interest that the U.C.L.A. fibrosarcoma had gradually decreased in viability during the previous year from 90–100 per cent takes in carrier animals.

TABLE 4

'FREE' AMINO ACIDS IN LIVERS OF CONTROL AND TUMOR-BEARING RATS

Experiment V			Amino acid (μg/mg of protein)			
Tumor	No. rats	Ethionine	Glycine	Methionine	Serine*	Threonine
Jensen (small)	7	none	53.5	4.78	56.9	18.4
Jensen (large)	17	none	62.3	8.30	83.0	33.2
Jensen (small)	16	0.25 per cent	60.8	5.78	62.9	25.0
Jensen (large)	10	0.25 per cent	65.9	8.35	82.7	37.0
Jensen (small)	18	0.50 per cent†	57.8	5.80	57.8	23.4
Jensen (large)	4	0.50 per cent†	67.0	9.51	99.8	31.9
None	15	none	45.6	5.24	55.9	16.5
None	15	0.25 per cent	86.0	11.8	113.0	44.2
None	14	0.50 per cent†	54.1	6.00	54.8	21.2

* Serine was determined by Camien and Dunn's improved unpublished method.
† 20 mg of ethionine/rat/day from the 14th to the 26th day.

At the time of Experiments II and III relatively few U.C.L.A. fibrosarcoma tumors normally failed to regress in carrier animals (Chart 2). In these experiments the administration of ethionine apparently interfered with the defense mechanism which was operating so efficiently in the control rats. This striking reversal of effect indicates that a metabolic antagonist, such as ethionine, may interfere with tumor resistance mechanisms under one set of conditions and with tumor growth under another. The marked increase in tumor necrosis, observed in Experiments I–III following the administration of ethionine, has been noted in greater or lesser degree in all experiments of this kind performed either with the U.C.L.A. fibrosarcoma or the Jensen sarcoma.

From studies with female rats in Experiment IV it was found that ethionine induced definite, but slight, retardation of tumor growth. The tumors of the treated animals were extremely necrotic with an orange discoloration, were soft and filled with a pink nonviscous fluid, and were commonly hemorrhagic and ulcerated. Most of the

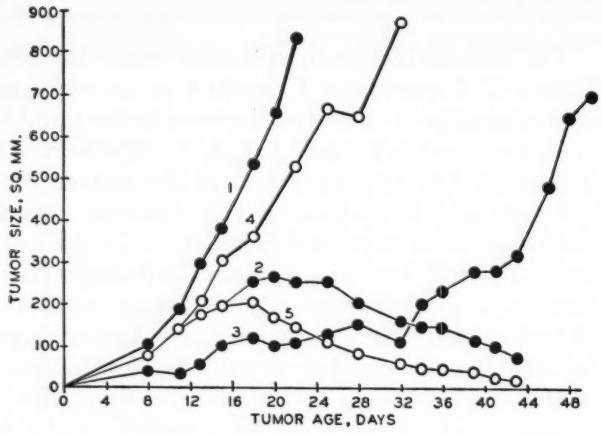

CHART 1 (Experiment I).—Curves showing the effect of ethionine on the growth of the U.C.L.A. fibrosarcoma in female rats. The notations are as follows:

● = Controls: No ethionine.
Curve 1: represents six rats; average tumor size at 36 days was 17,650 mm².
Curve 2: represents nine rats.
Curve 3: represents one rat.

○ = Experimental group; 0.5 per cent ethionine in diet from day 8 to day 20, 1.0 per cent ethionine in diet thereafter.
Curve 4: represents one rat; average tumor size at 43 days was 1,475 mm².
Curve 5: represents seventeen rats.

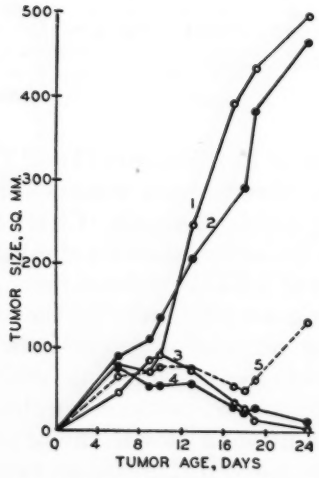

CHART 2 (Experiment II).—Curves showing the effect of ethionine on the growth of the U.C.L.A. fibrosarcoma in female rats. The notations are as follows:

● = Experimental diet contained 1.0 per cent ethionine.
○ = Control diet, no ethionine.
Curve 1: one animal.
Curve 2: seven animals.
Curve 3: ten animals.
Curve 4: seven animals.
Curve 5: one animal.

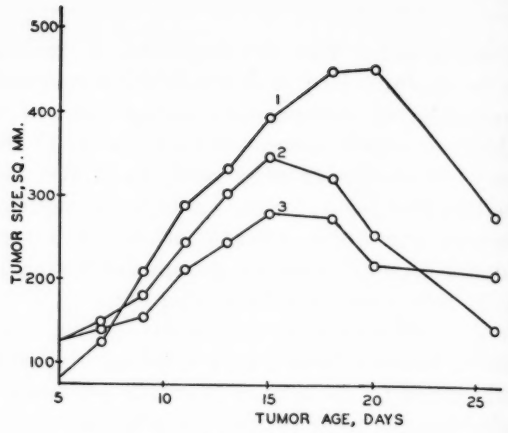

CHART 3 (Experiment V).—Curves showing the growth of Jensen sarcoma in male rats whose tumors regressed. The notations are as follows:

Curve 1: Control animals. Data represent 29 per cent of the total control animals.
Curve 2: Experimental animals; ethionine level was 0.25 per cent of the diet.* Data represent 62 per cent of the total animals.
Curve 3: Experimental animals; ethionine level was 0.50 per cent of the diet.* Data represent 82 per cent of the animals.

*Experimental animals were changed to control diet plus daily injections of 20 mg. of ethionine from 14th to 26th day.

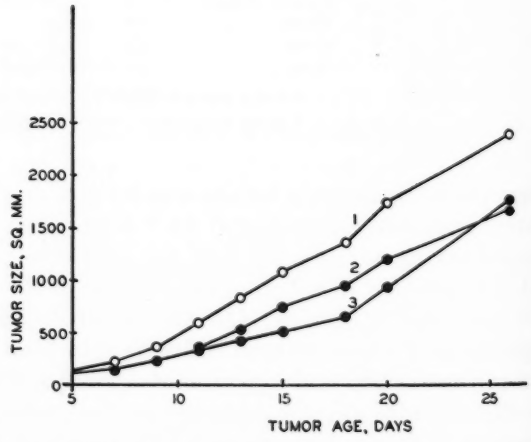

CHART 4 (Experiment V).—Curves showing the growth of Jensen sarcoma in male rats with nonregressing tumors. The notations are as follows:

Curve 1: Control animals. Data represent 71 per cent of the animals.
Curve 2: Experimental animals; ethionine level was 0.25 per cent of the diet.* The data represent 38 per cent of the animals.
Curve 3: Experimental animals; ethionine level was 0.50 per cent of the diet.* Data represent 18 per cent of the animals.

*Experimental animals were changed to control diet plus daily injections of 20 mg. of ethionine from the 14th to 26th day.

rats had died by the 26th day after implantation of the tumor, possibly from the effects of toxic products of necrosis.

In Experiment V the tumors regressed in 29 per cent of the control animals (Charts 3 and 4). Tumor growth was retarded by ethionine and the percentage of regressions increased with increase in ethionine administered. Tumors regressed in 62 per cent of the animals receiving 0.25 per cent ethionine and in 82 per cent of the animals receiving 0.50 per cent ethionine. Retardation only in the early stages of tumor growth resulted from the injection of 20 mg. of ethionine per day.

In Experiment VII the growth of well established Jensen sarcoma tumors (7 days old, area 300 mm.^{2}) was markedly retarded by high toxic dosages of ethionine (Chart 5). It was observed, however, that the growth rate of the tumor became nearly normal following cessation of ethionine administration. In many instances the tumors of ethionine-treated animals were reduced almost completely to a black, pulpy, fluid-filled sac. On the other hand, more than 50 per cent of the animals died because of the toxicity of ethionine at the high levels employed.

There was no significant difference between the percentages of total amino acids in the livers of nontumor control, untreated tumor and ethionine-treated tumor rats (see Table 3).[1] On the other hand (see Table 4), the "free" amino acids (calculated in terms of μg of amino acid/mg of liver protein) invariably were present in larger (from about 15 to 65 per cent) amounts in livers of animals with large, as contrasted to small, tumors. It may be noted further that the "free" amino acids were markedly elevated in livers of nontumorous rats receiving 0.25 per cent ethionine in the diet until the end of the experiment, while the "free" amino acid levels in the livers of nontumor-bearing rats receiving injections of only 20 mg. of ethionine per day during the latter half of the experiment were essentially the same as in the livers of the nontumorous controls. Apparently, in nontumorous rats ethionine causes an accumulation of "free" amino acids in liver by interfering with protein synthesis. In animals bearing large tumors, "free" amino acids (presumably from the more expendable muscle tissue) accumulate in the liver in response to the increased demand for blood proteins and other essential metabolites consumed in inordinate amounts by the fast-growing tumor.

As shown in Table 3, the liver weight of animals with large tumors is maintained proportional to body weight (carcass plus tumor), even though the weight of the carcass per se is considerably reduced. In harmony with this observation is the report of Yeakel and Tobias (10) that the total nitrogen content of rats bearing an adenocarcinoma is proportional to the total body weight.

A tumor regresses whenever the tumor tissue is no longer able to overcome the growth controlling or retarding influences of its environment. Whether or not regression occurs depends upon the size, age, and viability of the tumor as well as upon the potency of the natural or artificial inhibitors. It is the view of Mefferd *et al.* (4, 5) that early tumor

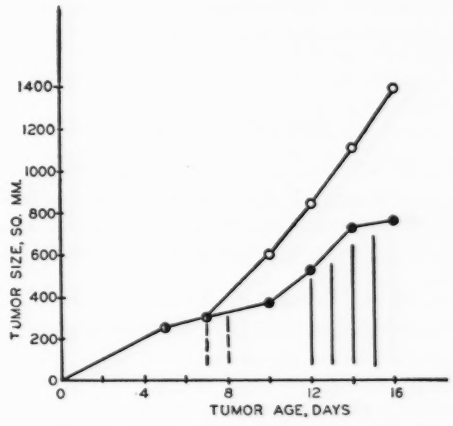

CHART 5 (Experiment VII).—Curves showing the effect of large doses of ethionine on the growth of Jensen sarcoma in female rats. The notations are as follows:

○ = Controls.

● = Experimental; dashed-bars indicate injections of 200 mg. of ethionine, solid-bars, 100 mg. of ethionine: the LD_{50} was apparently a cumulative dose of 400 mg. of ethionine.

regression is a function of 'intrinsic host resistance,' since they have shown that, within a certain size range, tumor regression and the size of the tumor implant are inversely related. The inhibition of tumor growth observed in the present experiments may be explained on this basis, although ethionine is an effective regressing agent only when it is acting in conjunction with strong natural inhibitors. It appears that there is a critical tumor size beyond which ethionine induces necrosis but is too toxic to be used as a regressing agent. This may be explained, possibly, by the lethal toxicity of the products of necrosis.

SUMMARY

Growth of the U.C.L.A. fibrosarcoma and the Jensen sarcoma in male and female rats of the Long-Evans strain has been investigated with and without the influence of DL-ethionine. Total and

[1] The fat content (data omitted to conserve space) of the livers of the ethionine-treated rats was nearly the same for animals with small or large tumors and was only about 25 per cent greater than that of the controls.

"free" amino acids (serine, threonine, methionine, arginine, glycine) have been determined in the livers of the rats by microbiological assay methods.

The administration of DL-ethionine retarded the growth of tumors, increased the tendency of tumors to regress, produced necrosis in all tumors examined, and, in nontumor-bearing animals, caused a marked increase in the "free" amino acids of the liver. There was also noted an increase in "free" amino acid content of livers of rats bearing large tumors. The weight of the liver was maintained proportional to the body weight (carcass plus tumor).

It was concluded that, when acting in conjunction with strong natural inhibitors, DL-ethionine is an effective regressing agent under the described experimental conditions.

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The Effect of 2,4-Dinitrophenol and of Fluoride on Oxidations in Normal and Tumor Tissues*

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INTRODUCTION

Earlier work from this laboratory (9, 11) has shown that the addition of fluoride permits isotonic homogenates of the Walker 256 carcinosarcoma and the Flexner-Jobling carcinoma to oxidize to an appreciable extent the various intermediates of the tricarboxylic acid cycle when supplemented with adenosinetriphosphate (ATP). From data on phosphate balance (11, 12), it has been suggested that the oxidative level is affected by the balance between high-energy phosphate breakdown and the uptake of inorganic phosphate into organic form. Thus, in the tumors where phosphate breakdown is very high, fluoride acts to preserve the respiratory rate by inhibiting the breakdown of high-energy phosphate (9, 11). Since the homogenates of several normal tissues do *not* need the addition of fluoride to demonstrate oxidation (8, 11, 12), it was thought worth while to investigate other tumors than those mentioned to observe whether the behavior in the presence of fluoride is characteristic of all tumor tissue.

Since it has been shown that 2,4-dinitrophenol (DNP) acts to stimulate the breakdown of high-energy phosphate (2, 4, 13), the present work was concerned with whether DNP further decreases the low oxidation rate in those tissue homogenates in which there is a stimulation by fluoride and whether it increases the oxidation of those tissue homogenates in which there is no effect by fluoride or where fluoride is inhibitory. In general, DNP and fluoride have been found to act somewhat antagonistically with respect to their effects on oxidation. Thus, the behavior of a homogenate of a tissue in the presence of DNP and of fluoride will roughly characterize that tissue as to its ability to break down high-energy phosphate and as to the ability of its oxidative phosphorylation mechanism to keep pace with this breakdown.

* Part of this paper was presented before the American Association for Cancer Research, New York, 1952 (20). This investigation was supported by a research grant (C-646) from the National Cancer Institute of the Public Health Service.

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MATERIALS AND METHODS

Tumors.[1]—The Flexner-Jobling carcinomas were 8–10-day-old subcutaneous transplants into 150-gm. female albino rats.[2] This tumor grows very rapidly, reaching a weight of about 1 gm. in 9–12 days.

The C3H mouse mammary carcinomas were subcutaneous transplants of a murine mammary adenocarcinoma of the small acinous type which arose spontaneously in the laboratories of Drs. Andervont and Barrett of the National Cancer Institute. The transplanted tumor contains the mammary cancer milk agent and is a slowly growing tumor.

The human tumors were obtained from the operating rooms of the University Hospitals, University of Wisconsin. They were immediately placed in saline at 0° C. following their excision, and the experiments were performed within about 1 hour.

The Novikoff rat hepatomas were 7-day-old intraperitoneal transplants of a hepatoma originally induced by feeding 4-dimethylaminoazobenzene. It is a very rapidly growing tumor and yields 10–15 gm. of non-necrotic tumor tissue in 7–14 days, depending on the size of the inoculum.

The mouse 98/15 hepatomas were subcutaneous transplants into C3H mice. This hepatoma grows slowly and requires about 4 months to reach a weight of 1 gm. However, it may become necrotic soon after it reaches this size.

The mouse adrenal cortical tumors were subcutaneous transplants of a tumor originally obtained by Dr. G. Woolley, Sloan-Kettering Institute, New York, following the castration of a female mouse of the CE strain. These tumors also grow very slowly and require 4–5 months to reach a weight of about 1 gm.

All the experimental animals were fed Friskie dog pellets.[3]

Freshly obtained, chilled tissues were homogenized in ice-cold isotonic sucrose to give a 10 per cent suspension. The mitochondria were prepared according to the method of Schneider and Hogeboom (16). The homogenization and centrifugations were carried out as quickly as possible in a cold room at 0–3° C., and the flask contents were also maintained at this temperature. The "standard" medium contained the following ingredients:[4] 30 μM phosphate buffer, pH 7.4; 9 μM $MgCl_2$;

[1] The authors are indebted to the following for the tumors used: Dr. G. A. LePage (Flexner-Jobling rat carcinoma), Dr. R. K. Boutwell (mouse mammary carcinoma), Dr. A. Curreri (human tumors), Dr. A. B. Novikoff, University of Vermont (transplantable rat hepatoma), Dr. G. Hogeboom, National Cancer Institute (98/15 mouse hepatoma), and Dr. H. P. Rusch (mouse adrenal cortical tumor).

[2] Obtained from the Holtzman-Rolfsmeyer Rat Co., Madison, Wis.

[3] Obtained from the Carnation Co., Milwaukee, Wis.

[4] ATP was obtained as the disodium salt and AMP as the free acid from the Pabst Laboratories, Milwaukee, Wis.;

4×10^{-2} μM cytochrome c; 3 μM ATP; 10 μM fumarate; and 20 μM pyruvate; enough solid sucrose to make the final mixture isotonic; and enough isotonic sucrose solution to compensate for variations in the amount of homogenate or mitochrondria added and give a final volume of 3.0 ml. The tissue homogenates or mitochondria were always prepared in isotonic sucrose. When fluoride was added, the final concentration was usually 0.01 M, and the flasks without fluoride contained sodium chloride at the same molarity (0.01 M). The concentration of DNP was varied over a wide range, but the molarity was always so low that the controls contained equivalent amounts of water. Inorganic phosphate was measured by a modification (18) of the Lowry-Lopez method (7). Oxygen uptake was measured by means of the conventional Warburg apparatus and incubations were at 38° C. unless otherwise noted.

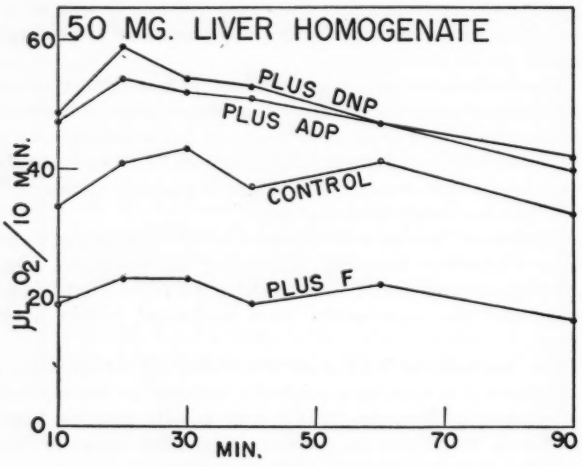

CHART 1.—The effect of DNP, fluoride, and ADP on oxidation by rat liver homogenate. Each flask contained the standard medium and 50 mg. rat liver homogenate. When added, DNP was at a final concentration of 3×10^{-5} M, and ADP at 2×10^{-3} M.

The pyruvic acid was vacuum-distilled and stored at 0–3° C. as a 1 M solution, which was neutralized with dilute KOH just before use.

RESULTS

In rat liver homogenates, DNP stimulated respiration while fluoride inhibited it (Chart 1). These effects are probably due to a DNP-stimulated breakdown of ATP to give phosphate acceptor in the form of adenosinediphosphate (ADP) and to an inhibition of this breakdown by fluoride (18, 19). The addition of ADP also stimulated respiration to about the same extent as did the addition of DNP. Chart 2 shows that additions of DNP, from a final concentration of 3×10^{-6} M to a final concentration of 6×10^{-5} M, progressively increased the respiration rate; 0.03 M fluoride had a quantitative effect equal to 0.01 M fluoride in inhibiting the rate. Thus, liver homogenates have a rate of ATP breakdown which is inadequate to saturate the oxidative capacity. This imbalance is even more pronounced in the mitochondria, since the respiratory rate of liver mitochondria can be stimulated three- to sevenfold by the addition of 3×10^{-5} M DNP (cf. 4, 19), an even greater extent than in the case of the whole homogenate. It would therefore appear that the other constituents of the liver cell—nuclei (cf. 12), microsomes (cf. 14), and perhaps supernatant fluid contain factors for the breakdown of ATP, but that these additional "ATPase" factors do not supply enough phosphate acceptor to saturate the oxidative system in liver homogenates. The addition of fluoride might be expected to have a greater effect on liver homogenates than on liver mitochondria, since the latter have a limiting rate of ATP breakdown even in the absence of fluoride. However, fluoride actually has a similar effect on each, possibly by inhibiting the adenylate kinase reaction (18).

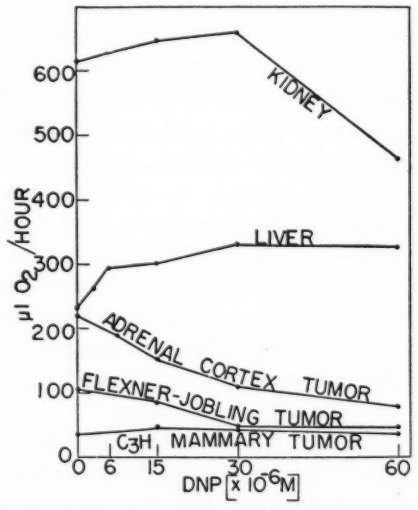

CHART 2.—The effect of DNP concentration on the oxidations by homogenates of kidney, liver, adrenal cortex tumor, Flexner-Jobling tumor, and C3H mouse mammary tumor. The conditions for the liver homogenate experiments are the same as given in Chart 1; for the kidney homogenate, the same as given in Chart 3; for the Flexner-Jobling homogenates, the same as given in Chart 4; for the C3H mouse mammary tumor homogenates, the same as given in Chart 10; for the adrenal cortex tumor homogenate, the same as given in Chart 8, except that the incubation temperature was 30° C.

The picture with kidney homogenates is slightly different (Chart 3); 3×10^{-5} M DNP has no effect initially, but after 1 hour it decreases the respiratory rate markedly, while 0.01 M fluoride has about the same inhibitory effect as in the case of liver.

Cytochrome c was obtained from E. R. Squibb and Sons through the courtesy of Dr. Asger Langlykke; DPN was obtained as the free acid from D. Groth and Dr. G. A. LePage; α-ketoglutaric acid was prepared by Dr. W. Ackermann; hexokinase was prepared from bakers' yeast, by an unpublished method of W. F. Loomis; other ingredients were commercial products used without further purification and converted to potassium salts if necessary.

Previously, it had been found that final DNP concentrations of from 6×10^{-6} to 6×10^{-5} M slightly increased the oxidative rates during the first 30 minutes, but that after this time the rate with 6×10^{-5} M DNP dropped very sharply, while the rates with 6×10^{-6} M, 1.5×10^{-5} M, and 3×10^{-5} M DNP declined more slowly. However, after 60 min. all rates were less than that of the control without added DNP (Chart 2). DNP at a concentration of 1.2×10^{-4} M decreased the rate 45 per cent in the first 10 min., and by 40 min. the respiration had been completely suppressed. Fluoride at a concentration of 0.03 M decreased the rate even more than 0.01 M fluoride. These results with DNP and with fluoride confirm earlier data of Pardee and Potter (8). The rates of phosphate breakdown and phosphate uptake are thus more nearly balanced in kidney than in liver homogenates, in that phosphate breakdown seems to be adequate enough to supply phosphate acceptor for the respiration (cf. 8). However, in kidney mitochondria 3×10^{-5} M DNP gave a threefold initial stimulation in the respiratory rate, with only slight decline in the rate after 90 min., and 0.01 M fluoride did not affect the rate at all. Thus, the other components of the kidney cell (cf. 8) seem to be more influential than the corresponding components of the liver cell in providing factors for bringing phosphate breakdown into balance with phosphate uptake.

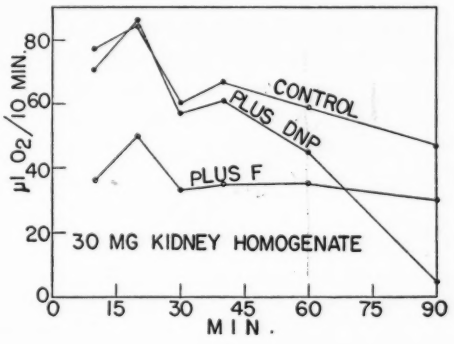

CHART 3.—The effects of DNP and fluoride on oxidation by kidney homogenate. Each flask contained the standard medium and 30 mg. rat kidney homogenate. When added, DNP was at a final concentration of 3×10^{-5} M.

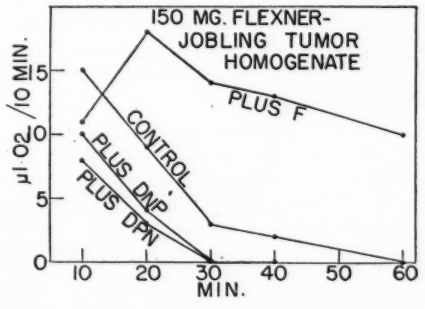

CHART 4.—The effect of fluoride, DPN, and DNP on oxidation by homogenates of Flexner-Jobling tumor. Each flask contained the standard medium and 150 mg. Flexner-Jobling carcinoma homogenate. When added, DNP was at a final concentration of 7.5×10^{-6} M and DPN at a final concentration of 0.002 M.

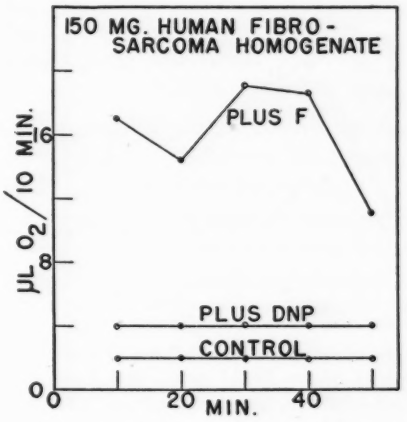

CHART 5.—The effect of fluoride and DNP on oxidation of human fibrosarcoma homogenate. Each flask contained the standard medium with cytochrome c omitted and 150 mg. of the human fibrosarcoma homogenate. When added, DNP was at a final concentration of 3×10^{-5} M.

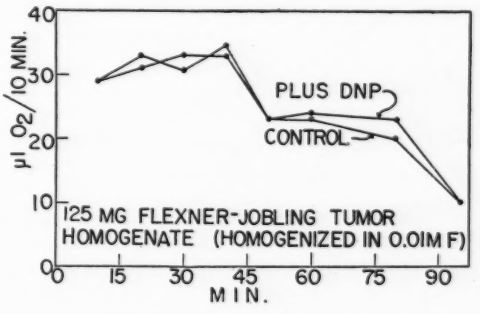

CHART 6.—The effect of DNP on oxidation by Flexner-Jobling tumor homogenized in fluoride. Each flask contained the standard medium and 125 mg. Flexner-Jobling carcinoma homogenate. The tumor was homogenized in isotonic sucrose solution made 0.01 M with respect to NaF. When added, DNP was at a final concentration of 3×10^{-5} M.

Charts 4–10 show the great dissimilarity of various tumors in their response to DNP and fluoride. For example, the Flexner-Jobling carcinoma (Chart 4) and the human fibrosarcoma (Chart 5) yield homogenates in which the rate of phosphate breakdown is much too great relative to the capacity of the tumor to regenerate high-energy phosphate oxidatively. Concentrations of DNP from 7.5×10^{-6} M to 6×10^{-5} M DNP greatly decreased the already low oxidative rate of the Flexner-Jobling tumor (Chart 2), while 0.01 and 0.03

M fluoride increased the rate to the same extent. When the Flexner-Jobling tumor was homogenized in the presence of 0.01 M fluoride, not only was the subsequent oxidative rate increased (cf. Charts 4 and 6), but the addition of DNP had no effect in accelerating phosphate breakdown. That the rate of phosphate breakdown is excessive relative to oxidation in the mitochondria from the Flexner-Jobling tumor was shown by experiments in which concentrations of DNP from 7.5×10^{-6} to 3×10^{-5} M decreased the already low mitochondrial respiratory rate. Also, 0.01 M and 0.03 M fluoride were as effective in increasing the rate as they were in the case of the whole homogenate. The high rate of ATP breakdown by Flexner-Jobling carcinoma mitochondria is also shown in Table 1; these data show that the addition of Flexner-Jobling carcinoma mitochondria stimulates the respiratory rate of liver mitochondria (cf. 8). This stimulation is presumably caused by an activation of ATP breakdown to provide enough phosphate acceptor for the oxidative system of the liver mitochondria, since this synergistic effect of the tumor mitochondria is greatly reduced by the addition of fluoride and since the effect can be reproduced by DNP.

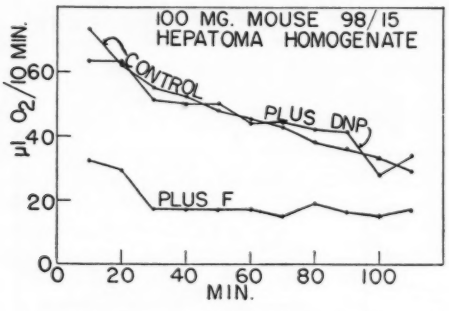

CHART 7.—The effect of DNP and fluoride on oxidation by homogenate of mouse 98/15 hepatoma. Each flask contained the standard medium, except that 6 μM ATP were added instead of 3 μM ATP, and 100 mg. of the mouse 98/15 hepatoma homogenate. When added, DNP was at a final concentration of 3×10^{-5} M.

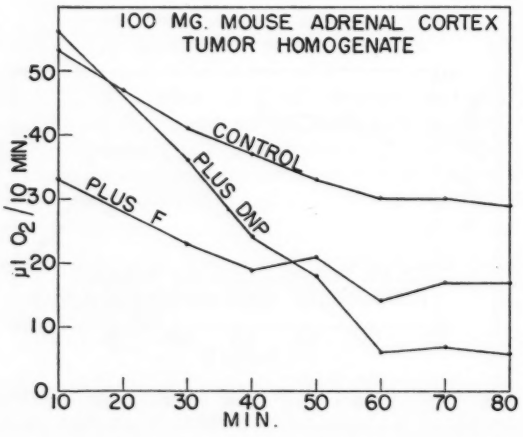

CHART 8.—Effect of DNP and fluoride on oxidation by mouse adrenal cortex tumor homogenate. Each flask contained the standard medium with cytochrome c omitted and 100 mg. of the mouse adrenal cortex tumor homogenate. When added, DNP was at a final concentration of 3×10^{-5} M.

Between the 8th and 11th days after transplantation of the Flexner-Jobling carcinoma, the tumors approximately doubled their weight every 36 hours. In experiments on homogenates from pairs of 8-, 9-, 10-, and 11-day-old tumors that developed from matched implants, there was no detectable change in the effects of DNP and of fluoride on the respiration. Also, in this series of experiments there was no clear-cut effect of added diphosphopyridine nucleotide (DPN) (0.002 M) on

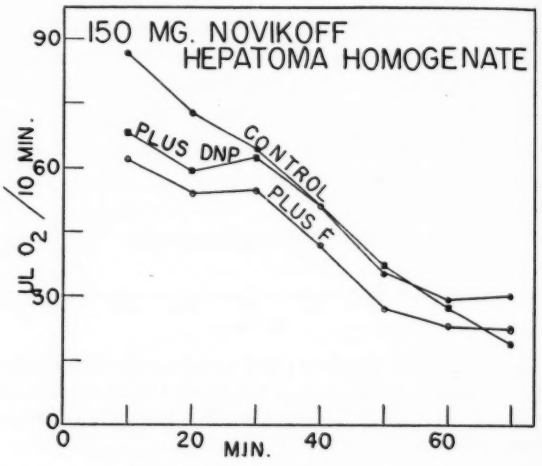

CHART 9.—Effect of DNP and fluoride on oxidation by Novikoff hepatoma homogenate. Each flask contained 150 mg. of the Novikoff rat hepatoma homogenate and the standard medium with cytochrome c omitted and with 6 μM ATP added instead of the 3 μM ATP. When added, DNP was at a final concentration of 3×10^{-5} M.

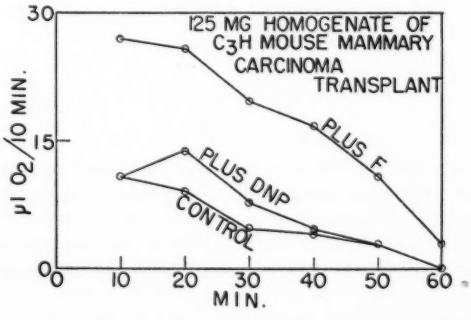

CHART 10.—Effect of DNP and fluoride on oxidation by homogenate of C3H mouse mammary carcinoma. Each flask contained the standard medium, except that the substrates were 5 μM fumarate and 10 μM pyruvate, and 125 mg. of the C3H mouse mammary carcinoma homogenate. When added, DNP was at a final concentration of 3×10^{-5} M.

the respiratory rate of these tumor homogenates. In most instances there was an inhibition of the already low respiratory rate (Chart 4), and the addition of fluoride did not relieve the inhibition by DPN. The oxidative rate of the mitochondria from this tumor was also inhibited by DPN. In only one instance was there an appreciable stimulation of the respiratory rate of the homogenate by DPN. It is concluded that the effects observed are independent of the age of the tumors within the limits noted and are not due to necrosis.

In contrast, three other tumors, the mouse hepatoma 98/15 (Chart 7), the adrenal cortical tumor (Chart 8), and, to a lesser extent, the Novikoff hepatoma (Chart 9) resemble kidney in their responses to DNP and to fluoride. All these tumor homogenates had a respiratory rate that was much higher than that of the Flexner-Jobling tumor, that was inhibited by fluoride, and that showed a response to DNP very unlike that of the Flexner-Jobling tumor and more similar to that of kidney. DNP at a concentration of 7.5×10^{-6} M had very little inhibitory effect on the high oxidative rate of the adrenal cortical tumor homogenate, but higher concentrations of DNP (up to 6×10^{-5} M) had increasingly larger inhibitory effects on the respiration (Chart 2).

The C3H mouse mammary carcinoma (Chart 10) gave a unique response, since both fluoride and, to a much lesser extent, DNP stimulated the respiration; 1.5×10^{-5} M DNP gave approximately the same small increase in rate as did 3×10^{-5} M DNP, while 6×10^{-5} M DNP had no effect (Chart 2). Also, with this tumor, *DPN* stimulated the respiration of the homogenate, not so much as fluoride, but more than DNP. The significance of these effects is lessened by the low magnitude of oxygen uptake in all conditions.

TABLE 1

EFFECT OF FLEXNER-JOBLING TUMOR MITOCHONDRIA UPON RESPIRATION OF LIVER MITOCHONDRIA

Each flask contained the standard medium with cytochrome c omitted and either mitochondria from 300 mg. wet weight rat liver, or mitochondria from 600 mg. wet weight Flexner-Jobling carcinoma, or mitochondria from 150 mg. wet weight rat liver plus mitochondria from 300 mg. wet weight Flexner-Jobling carcinoma. When added, DNP was at a final concentration of 3×10^{-5} M and F at 10^{-2} M. The theoretical oxygen uptake was obtained by adding one-half the rate with the liver mitochondria to one-half the rate with tumor mitochondria.

	Microliters O_2/hr		
	Control	Plus F	Plus DNP
Liver mitochondria	282	221	681
Flexner-Jobling mitochondria	40	38	14
½ Liver mitochondria plus ½ Flexner-Jobling mitochondria	214	144	570
Theoretical	161	130	347

Table 2 shows that most metabolites involved in the tricarboxylic acid cycle, with the exception of succinate, are not oxidized very well by the Flexner-Jobling carcinoma or the C3H mouse mammary tumor homogenates. However, all the substrates except citrate and cis-aconitate were oxidized to a much greater extent in the presence of fluoride.

TABLE 2

EFFECT OF SUBSTRATE ON OXIDATION OF FLEXNER-JOBLING AND C3H MOUSE MAMMARY TUMOR HOMOGENATES

Each flask contained the standard medium except for the substrate and either 150 mg. Flexner-Jobling tumor homogenate or 125 mg. of the C3H mouse mammary carcinoma homogenate. All substrates were added in 15 μM quantities except pyruvate (10 μM) and fumarate (5 μM).

		Microliters O_2/hr*	
Tumor	Substrate	Plus F	Minus F
Flexner-Jobling	Succinate	95	72
	Alpha-ketoglutarate	57	17
	Oxalacetate	49	17
	Cis-Aconitate	7	5
	Citrate	6	5
	Pyruvate+fumarate	75	28
	Glutamate	57	15
C3H mouse mammary	Pyruvate+fumarate	104	35
	Citrate	20	29

* Minus endogenous respiration.

TABLE 3

OXIDATIVE PHOSPHORYLATION IN THE FLEXNER-JOBLING HOMOGENATE

Each flask contained 125 mg. Flexner-Jobling tumor homogenate and the standard medium with cytochrome c omitted and with 6 μM AMP substituted for the ATP. Incubated for 10 min. at 30°.

Additions	O_2 μ atoms	P μM	P/O*
None	−2.3	+0.60	
0.01 M NaF	−2.2	−4.27	0.95
Hexokinase, glucose (11 μM)	−1.6	−0.14	0.45
0.01 M NaF, hexokinase, glucose	−1.65	−4.27	1.3

* Corrected for period of equilibration.

Tables 3 and 4 show that, under appropriate conditions, tumors as different as the Flexner-Jobling carcinoma, which has a very low oxidative rate in the absence of fluoride, and the 98/15 mouse hepatoma, which has a high oxidative rate that is inhibited slightly by fluoride, can give a net phosphate uptake with a P/O ratio which is lower than that obtained with normal tissue under similar conditions. In both cases, the incubation temperature was lowered to 30° C. to reduce the effects of side reactions draining off high-energy phosphate.

The incubations were limited to short time periods, so that, as can be seen in Table 4, the coupling of phosphorylation to oxidation would not be disrupted by the longer time periods. In the case of the Flexner-Jobling tumor homogenate, it can be seen that only in the presence of fluoride was there a net esterification of inorganic phosphate. Although the 98/15 hepatoma homogenate had a high oxidative rate in the absence of fluoride, the addition of fluoride increased the already high phosphate uptake, indicating that there is some phosphate loss even with this tumor. It is interesting that with the addition of glucose without hexokinase to the tumor homogenate there was a greater net phosphate uptake, with or without fluoride, without any increase in respiration, indicating hexokinase activity (cf. 6). These data

TABLE 4

OXIDATIVE PHOSPHORYLATION IN THE 98/15 MOUSE HEPATOMA HOMOGENATE

Each flask contained 130 mg. mouse 98/15 hepatoma homogenate and the standard medium with cytochrome c omitted and with 12 μM AMP substituted for the ATP. Incubation at 30°.

Additions	10 min.			30 min.		
	O_2	P		O_2	P	
	μ atoms	μM	P/O*	μ atoms	μM	P/O*
None	−5.25	− 9.2	0.88	−15.9	− 9.1	0.43
0.01 M NaF	− 4.5	−12.7	1.41	−12.2	−15.1	0.93
Glucose (16.5 μM)				−15.2	−20.5	1.01
0.01 M NaF, glucose				−13.7	−31.6	1.74

* Corrected for period of equilibration.

showing differences between the 98/15 hepatoma and the Flexner-Jobling carcinoma are supported by earlier findings in the literature. R. Kielley (3) found that, in the *absence* of fluoride, the mitochondria of the 98/15 hepatoma would esterify inorganic phosphate in the presence of hexokinase with a P/O ratio of approximately 2 when α-ketoglutarate was oxidized, while Williams-Ashman and Kennedy (27) found that, in the *presence* of fluoride and hexokinase, the particulate matter of the Flexner-Jobling tumor would esterify inorganic phosphate when succinate was the substrate, with a P/O ratio of 0.7.

DISCUSSION

It is clear from the experimental data that the six tumors tested gave different patterns of oxidative response to DNP and to fluoride. There seems to be no correlation between any gross observations on the rates of growth of the various tumors and the oxidative responses of their homogenates in the presence of DNP or fluoride.

The possibility of such correlations may well be overshadowed by the influence of factors such as a residual resemblance to the tissue of origin and the species of animal used. Thus, there may be an alteration common to all these tumors, but it may fail to result in a consistent pattern of response to DNP and fluoride because of the differences in function and in enzymatic constitution of the tissues of origin of these tumors.

It might be argued (21) that, in the process of homogenizing the various tumors, the stability of the bound complexes is reduced more in one tumor than in another and that the responses to DNP and to fluoride reflect only a secondary effect resulting from the disruption of the organization of the cell. However, this possible differential disruption by homogenization would still reflect a difference among the various tumors in the stability of the oxidative phosphorylative system, and would also reflect a difference between most normal tissues (except spleen and thymus) and tumors in this respect (cf. 3). In addition, the great differences in oxidative response among the tumors tested here must be emphasized. The Flexner-Jobling carcinoma and the Novikoff hepatoma, which under our conditions have approximately the same high growth rate and thus synthesize approximately equal amounts of tissue per unit time, exhibit marked differences in the oxidative responses to fluoride and to DNP. Another difference among tumors is illustrated by the difference in the P/O ratios of the Flexner-Jobling carcinoma and the mouse 98/15 hepatoma when fluoride was added (cf. Tables 3 and 4) and in the great variation among P/O ratios obtained with the particles of various tumors by Williams-Ashman and Kennedy (27) when hexokinase was used as a trapping agent for esterified phosphate.

As a corollary to the difference in stability of the oxidative phosphorylative system between various tumors and normal tissues is the DPN requirement exhibited in a number of tumors as noted by Weinhouse and his group (21, 25, 26), by Williams-Ashman and Kennedy (27), and by R. Kielley (3). This requirement is not exhibited by kidney or liver under the same conditions (3, 21, 26). In nearly all these tumors the leakage of DPN

or its disruption from a bound site can be repaired by the addition of DPN, but in the Flexner-Jobling carcinoma the addition had no effect on homogenate or mitochondrial oxidation, although it has been reported (27) that even in this tumor the addition of DPN stimulated the oxidation of glutamate.

It has been reported that the individual enzymes necessary for the complete oxidation of any member of the tricarboxylic acid cycle were contained in all of the tumors tested (21–24). Also, it is clear that many of these tumors can oxidize various substrates of the tricarboxylic acid cycle to an appreciable extent if DPN is added (3, 24–26). However, it has been found that α-ketoglutarate dehydrogenase and aconitase activities were lower in tumor tissue than in normal tissue (24), that both succinoxidase and cytochrome oxidase were lower in mouse hepatoma than in mouse liver (17), and that 98/15 hepatoma has a much lower DPNH dehydrogenase activity than does normal mouse liver (5).

It has been stated (25, 26) that in those tumors where oxidation was low in comparison to normal tissue the oxidative activities of these tumor mitochondria on a per milligram N basis were of the same magnitude as those of three normal tissues studied. In contrast is a comparison between the 98/15 mouse hepatoma and mouse liver. Schneider and Hogeboom (17) state that the total succinoxidase and cytochrome oxidase activities of the liver mitochondria were over 5 times that of hepatoma mitochondria[5] and that the specific activity of these enzymes (on a per milligram N basis) of the liver mitochondrion was 2.5 times that of the tumor mitochondrion. Therefore, the decreased amount of mitochondrial N/mg wet weight of tissue in the hepatoma, as compared to the normal liver, still does not account for the decreased activity in the hepatoma. Thus, while it would appear that in some tumors the decreased amount of mitochondrial-bound enzyme per unit of cellular N can be attributed to the lowered content of mitochondria (25, 26), in other tumors there is an absolute decrease in some of the enzymes associated with mitochondria (17). At any rate, in both these cases the amount of enzyme per unit of tumor cell N, or, in other words, the enzymatic capacity of the cell, is reduced to a fraction of what it is in homologous normal tissues.

The capability of a tissue to be stimulated by DNP or by fluoride is also a measure, in a sense, of the oxidative capacity of the tissue. For example, the stimulation of the oxidation of some tumors by the addition of fluoride or by the addition of DPN may mean that the oxidative capacity of the tumor is not sufficient to regenerate the lost co-factors, such as DPN or ATP. Also, the ability of some tumor oxidations to be inhibited by fluoride and to be unaffected or even stimulated by DNP may mean that in these cases the tissue is operating at a level of oxidation which is sufficient to regenerate the co-factors. Thus, it would appear that the wide variation in oxidative capacity among tumors is but a reflection of the wide variation existing among normal tissues. Perhaps even the greater lability of the phosphorylating mechanism in some tumors and not in others is but a reflection of what a comparison among normal tissues has shown and of the fact that these variations must be ascribed to a residual enzyme pattern derived from the tissue of origin.

From these studies it seems clear that the conclusions reached from metabolic studies on any given tumor cannot be generally assumed to hold for other tumors. Thus, investigators are faced with the task of finding the strategic metabolic steps in the growth process in the presence of a variety of enzymatic patterns that represent in varying degrees the multiplicity of enzyme patterns seen in normal growing and nongrowing tissues. Although we have repeatedly found a lower oxidative capacity in tumor tissues in comparison with the tissues of origin, particularly in the case of liver,[5] the present studies make generalizations on this point inadvisable, largely because of the inability to provide adequate data on the tissues of origin of all the tumors studied. It is equally apparent that the recently advanced *opposite* generalization that the oxidative enzyme pattern of tumors is no different from that of normal tissues is also untenable, because here the reservation is not based upon a lack of suitable controls, but countered by positive findings in the case of some of the hepatomas.

It would appear that the next step is to study the metabolism of a variety of tumors under conditions of actual growth in the host and to ascertain the balance between as many alternative catabolic and anabolic pathways as possible, on the assumption that uncontrolled growth involves the dominance of certain anabolic pathways over the catabolic pathways *for the same substrates*. Perhaps these metabolic switches can eventually be found, even though the important reactions may occur in a variety of over-all enzyme patterns. Possible support for this view was obtained in the

[5] Reif and Potter also found (unpublished) that the succinoxidase activity/mg wet weight tissue is about one-fifth as great in 98/15 hepatoma as in mouse liver and about one-fourth as great in various azo-dye induced hepatomas as in rat liver.

studies with fluoroacerate (10) in which one of the possible interpretations was that tumors do not activate acetate effectively. This interpretation has been supported by subsequent studies with acetate-1-C^{14} (1). In attempting to find a metabolic pathway that might be characteristic of all tumors, studies with glucose-1-C^{14} *in vivo* have been started in this laboratory (15), and it has been found that derivatives of glucose are extensively used in anabolic reactions. Thus, it may be possible eventually to study the important shunts in glucose metabolism even in the presence of the divergent enzyme balances described in the present report.

SUMMARY

The capacity and the lability of the coupled oxidative phosphorylation systems of liver, kidney, and of seven different tumors have been investigated with the aid of DNP and of fluoride, which act to stimulate and to inhibit, respectively, the net breakdown of high-energy phosphate. No over-all oxidative pattern common to tumor homogenates has been observed. The great difference in oxidative response among various tumors has been emphasized, and the results have been considered to represent the contribution of the tissue of origin to the enzyme pattern of the tumor tissues, and to reflect the multiplicity of enzyme patterns seen in normal growing and nongrowing tissues. The significance of the findings was discussed in relation to the problem of finding the balance between anabolic and catabolic pathways that involve the same substrates.

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In Vitro Demonstration of an Antigen in Red Blood Cells of C3H Mice*

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In previous experiments with the spontaneous C3H mouse mammary tumor it was observed that antisera produced in rabbits with filtrates from this tumor inhibited the hemolytic effect of 10 per cent saline suspensions of mammary tumors on mouse red blood cells (2). This inhibition was achieved only by sensitization of the mouse red blood cells, which in the nonsensitized state were hemolyzed by the tumor suspension. It was not achieved by mixing the antitumor serum with the tumor suspensions. The inhibition produced by the antimammary tumor serum was, on the average, 8 times greater than that produced by normal rabbit serum. These findings suggested further studies with antisera produced in rabbits with other mouse tumor filtrates, to determine whether this inhibition of hemolysis by suspensions from mammary tumors is specific for the antimammary tumor serum. Accordingly, experiments were made with antitumor rabbit sera prepared with two different brain tumors induced in mice with methylcholanthrene (6): (*a*) an undifferentiated malignant glioma grown in the C3H mouse strain (which is susceptible to spontaneous mammary tumor) and (*b*) an ependymoma grown in the C57BL strain (which is resistant to mammary tumor).

It has also been found that there are striking differences in the hemagglutinating and hemolytic behavior between the red blood cells of the C57BL mice, which are mammary tumor-resistant, and those of the C3H mice, which are susceptible (1, 2). And, most significantly, there is a difference between the red blood cells from tumor-bearing and those from nontumor-bearing animals of comparable ages (over 1 year) of the C3H strain. Thus, the questions arise: (*a*) Does this difference in the hemagglutinating and/or hemolytic behavior represent a difference in the antigenic constitution of these red blood cells; and (*b*) is there a relationship between the antigenic constitution of these red blood cells and the tumor? To investigate these points, rabbit antisera were prepared with red blood cells from nontumorous C57 mice, from nontumorous C3H mice, and from C3H mice bearing spontaneous mammary tumors. These antisera were tested in the same manner as the antitumor rabbit sera for inhibition of the hemolytic effect of suspensions of mammary tumor on mouse red blood cells.

MATERIALS AND METHODS

C3H and C57BL mice from our own breeding colony were employed for tumor growth and for bleeding as previously described (1). The tumors used for immunization of rabbits were:

1. C3H(19), an undifferentiated, malignant glioma originally induced in a C3H mouse with methylcholanthrene and carried on by subcutaneous transplantation in the C3H strain. This tumor grew rapidly and killed the mice in an average of 30 days after transplantation. The tumor was employed in the 63d–65th passage.

2. C57(26), an ependymoma originally induced with methylcholanthrene in a C57BL mouse and carried on by subcutaneous transplantation in the C57BL strain. This tumor was used in the 55th passage. It had a much slower rate of growth than the C3H(19) tumor but finally killed the mice in an average of 100 days.

3. Spontaneous mammary tumors from C3H mice were used for the control sera.

Silk filtrates of all tumors were prepared as previously described (2).

The erythrocytes used for immunization were obtained from (*a*) nontumorous C3H mice, (*b*) nontumorous C57BL mice, and (*c*) C3H mice with spontaneous mammary tumors in various stages of growth. Animals of both sexes were employed and, with few exceptions, at 13–20 months of age. Ten per cent red blood cell suspensions were used. The red blood cells were always kept on ice and employed within 60–90 minutes after bleeding.

The rabbits were immunized with either the tumor filtrates or the red blood cell suspensions in exactly the same manner as to dose and interval of injection: One intravenous injection of

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1 cc. of tumor or red blood cell material was followed by four intraperitoneal injections of 3 cc. However, with the ependymoma C57(26) filtrates, one intravenous and only three intraperitoneal injections were given because of lack of material. Blood from the rabbits was drawn from the heart 3–4 weeks after the last injection. The complement of the sera was inactivated at 56° C. for 30 minutes, and the sera were then stored at −20° C. for various lengths of time. The control sera from normal rabbits were treated in the same manner.

Inhibition of the hemolytic effect of suspensions from mammary tumors on mouse red blood cells sensitized with the rabbit antisera was determined as previously reported (2). The red blood cells were sensitized in the following manner: Two-tenths cc. of red blood cell suspension was added to 0.2 cc. of twofold serial saline dilutions of antitumor rabbit serum or anti-RBC serum and to corresponding dilutions of normal rabbit serum, respectively. The mixtures were incubated for 60 minutes at 37° C. and then for 90 minutes at 4° C. They were then centrifuged for 4 minutes at 2,000 r.p.m., and the supernatant sera were discarded. The red blood cells were taken up in 3 cc. of cold sterile saline, centrifuged again for 4 minutes at 2,000 r.p.m., and freed from the saline. Afterwards, the hemolytic effect of the tumor suspensions was tested on the sensitized mouse red blood cells. Ten per cent tumor suspensions were freshly prepared with cold saline at 15° C., and the supernatant material employed in the tests was obtained by centrifugation at a radius of 4 inches and at 3,250 r.p.m. for 6 minutes at room temperature. In control tests, saline instead of tumor suspension was added to the sensitized red blood cells. Results were evaluated by titer according to the highest serum dilution causing inhibition of hemolysis with the tumor suspension. Readings were made after 3–5 hours (earlier than in the previous experiments), after 15–18 hours, and after 48 hours.

RESULTS

The results are summarized in Chart 1. It is evident that the inhibition of the hemolytic effect of suspensions of mammary tumors on mouse red blood cells was not specific for the antimammary tumor serum. Inhibition was approximately the same when the mouse red blood cells were sensitized with antitumor rabbit sera prepared with glioma C3H(19) or ependymoma C57(26) filtrates, or with antitumor rabbit sera prepared from C3H mammary tumors. The titer of inhibition was in the range of 32–64, with some minor deviations to either side, and corresponded to the titer observed previously with antimammary tumor rabbit sera. If very slight differences are considered, the anti-C3H(19) serum had a somewhat better inhibitory effect than the antimammary tumor serum; anti-

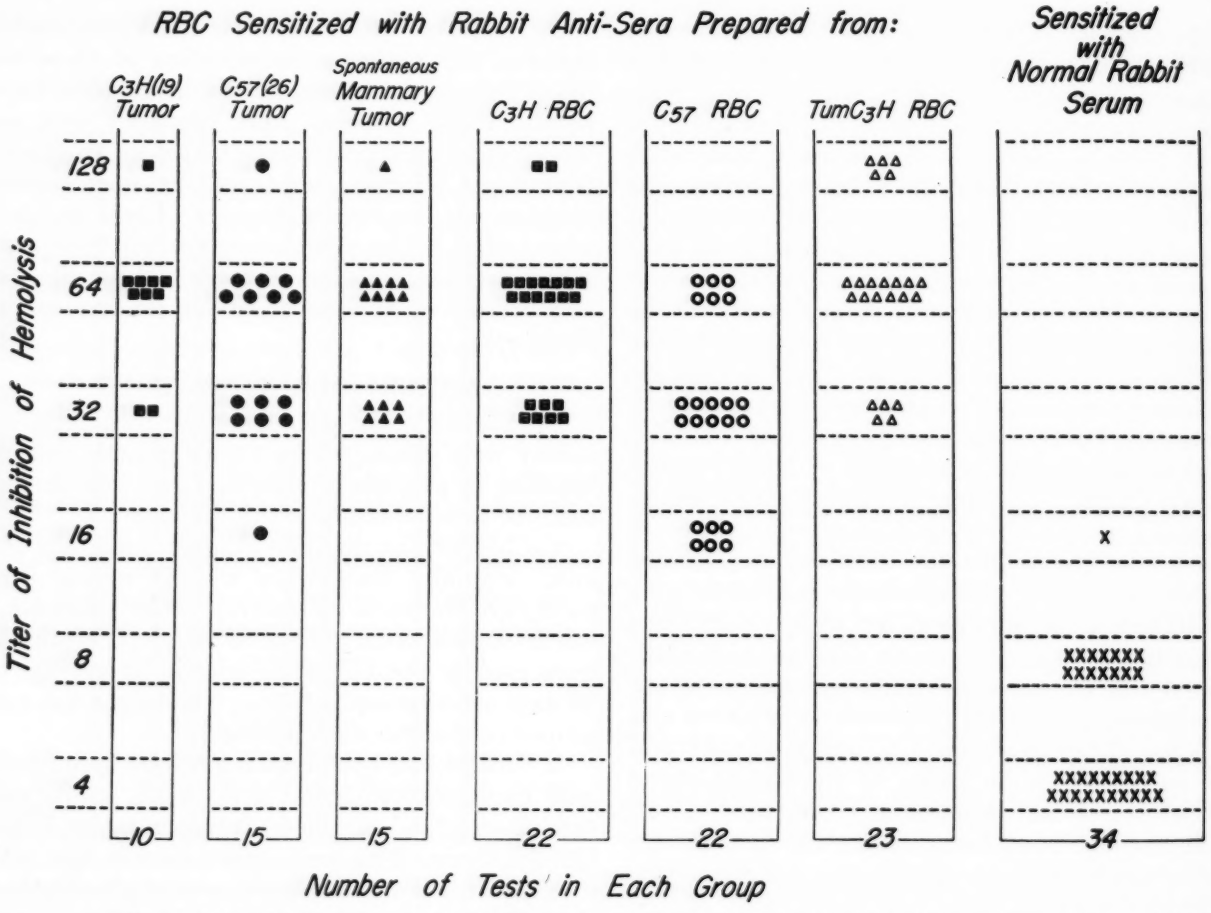

CHART 1.—Titer of inhibition of hemolysis of mouse red blood cells by mammary tumor suspensions after sensitization of the red blood cells with rabbit antisera. Each symbol indicates one test read after 3-5 hours.

C57(26) serum, in one instance, had an inhibitory titer of only 16.

Sensitization of the mouse red blood cells with anti-red blood cell sera inhibited the hemolytic potency of tumor suspensions differently according to the origin of the red blood cells used for immunization. After sensitization with anti-C3H red blood cell sera, the titer of inhibition was 32 in seven instances, 64 in thirteen instances and 128 in two instances. After sensitization with anti-C57 red blood cell serum, the titer of inhibition was 16 in six instances, 32 in ten instances and 64 in six instances. After sensitization with anti-C3H red blood cell serum (produced with red blood cells from C3H mice bearing spontaneous tumors and designated Tum C3H), the titer of inhibition was 32 in five instances, 64 in thirteen instances, and 128 in five instances. In control tests, after sensitization of the mouse red blood cells with normal rabbit sera, the titer of inhibition was 4 in nineteen instances, 8 in fourteen instances, and 16 in one instance. Thus, it is evident that the titer of inhibition of hemolysis produced by anti-C3H red blood cell sera was about the same as that of the antitumor sera tested. Sensitization with anti-C57 red blood cell sera, however, produced a definitely lower titer of inhibition. Sensitization with the anti-Tum C3H red blood cell sera gave the strongest inhibition observed with any antisera employed in this series, obviously even stronger inhibition than with the antitumor sera.

In each test, red blood cells from nontumorous C3H and C57 mice and from C3H mice with spontaneous mammary tumors were used for sensitization. There was no remarkable difference between the red blood cells at readings after 3–5 hours. At readings after 15–18 hours, sensitized C57 and Tum C3H red blood cells reacted about the same, but sensitized C3H red blood cells frequently showed a twofold lower titer.

In the control series, wherein saline instead of tumor suspension had been added, hemolysis was not observed after 24 hours. After 48 hours hemolysis of C3H red blood cells occurred with higher serum dilutions. Occasionally it also occurred with Tum C3H red blood cells, but not with C57 red blood cells.

DISCUSSION

From the data presented it is evident that the inhibition of the hemolytic effect of suspensions from mammary tumors on mouse red blood cells is not specific for the antimammary tumor serum alone. Inhibition has been observed to the same degree with antitumor sera prepared with filtrates from tumors of different origin and also with antitumor sera prepared with filtrates from a tumor of a different mouse strain.

With anti-erythrocyte sera the findings are even more striking. The anti-C3H red blood cell sera caused inhibition of the hemolytic effect of tumor suspensions on mouse red blood cells in the same manner and to the same degree as antitumor sera. By contrast, the anti-C57 red blood cell sera showed definitely less inhibition than the anti-C3H red blood cell sera and all the antitumor sera tested. This is significant, because four rabbits were employed in the preparation of each of the antisera and also because, in the paired tests performed with C3H and C57BL red blood cell antisera, the titer was invariably lower with the antisera of the latter mouse strain. These findings indicate that the C3H red blood cells have an antigen in common with or related to the tumor which the C57 red blood cells do not have. The presence of an antigen in mouse red blood cells of tumor-susceptible animals has been suggested by Gorer (5). He found that the red blood cells from the A strain of mice have at least one antigen in common with normal and neoplastic tissues of this strain. Recently, while the present work was in progress, Barrett contributed more data on this problem (3, 4). In mice with differing genetic constitution, he induced resistance to tumor growth by prior injection of red blood cells derived from the strain in which the tumor had originated. Thus, the results of the present study, although obtained with antisera produced with crude antigens, confirm the findings of Gorer and Barrett from another angle. Attention should be drawn, however, to the fact that in the current experiments the presence of an antigen related to the tumor was demonstrated *in vitro, without tumor transplantation* into the animals. This approach may offer a broader avenue for the investigation of tumor antigens in general.

Finally, the question arises whether the antigen found in the C3H red blood cells but not in the C57 red blood cells is related to the hemolytic factor observed with the C3H but not with the C57 erythrocytes. This question needs further investigation. One fact must be borne in mind: namely, that there is a relatively high titer of inhibition of hemolysis by antisera prepared with erythrocytes from tumor-bearing C3H mice, and only a limited hemolysis of the erythrocytes of these animals with dilutions of normal rabbit serum. Therefore, it seems unlikely that there is a relationship between the antigen and the hemolytic factor. Theoretically, however, it may be assumed that red blood cells from tumor-bearing mice acquire an antibody against the tumor in addition to the hemolytic factor which they possess. This anti-

body can act like the one which inhibits hemolysis of the red blood cells by tumor suspensions. Thus, the difference between the red blood cells from tumorous and nontumorous C3H mice would not depend so much upon a difference in the antigenic constitution as on the antibody content.

SUMMARY

1. Antitumor rabbit sera prepared with filtrates from glioma C3H(19), from ependymoma C57(26), and from spontaneous mammary tumors inhibit the hemolytic effect on erythrocytes by mammary tumor suspensions in approximately the same titer.

2. Anti-C3H red blood cell sera produce the same titer of inhibition as these antitumor sera.

3. Anti-C57 red blood cell sera have a slightly lower inhibitory effect than anti-C3H red blood cell sera and antitumor sera.

4. Antisera prepared with red blood cells from C3H mice with spontaneous mammary tumors produce the strongest inhibition.

5. The implications of these findings obtained by *in vitro* experiments are discussed.

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The Mammary Tumor Agent in Extracts of Frozen and Unfrozen Mammary Cancers*

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Gye and his associates (15–17, 20, 21, 24) have reported on the development of mouse tumors, either sarcomas or carcinomas, at the subcutaneous site of injection of either frozen and/or frozen-dried tumor suspensions. They interpreted their data to imply that the tumors were induced because of the action of a virus upon the normal cells, and the virus they called "the continuing cause of cancer." Recently, this theory has been applied to certain clinical problems (20, 21, 24).

In the case of mammary cancer, Mann (20, 21, 24) stated that the tumors develop because of the "selective infectivity" of the mammary tumor agent, made "active" because of freezing, when the active virus encounters a tubule of the normal mammary gland (20). The tumors appeared within a matter of days, or at most a few weeks, following the subcutaneous injection of the frozen tumor mince, but few mammary tumors appeared after the intraperitoneal administration of the thawed material.

This report is concerned with the tumor-producing activity of the mammary tumor agent obtained from centrifugates of frozen or unfrozen transplanted mammary carcinoma and with the tumors which arose following the injection of the thawed tumor mince. Preliminary data, tabulated 76–185 days after the administration of these extracts, have been reported (5).

TABLE 1

ASSAY FOR THE MAMMARY TUMOR AGENT IN FRESH AND FROZEN TISSUE OF THE AZF_1 MAMMARY CARCINOMA, NO. 8415A

A tumor which developed following the injection of the thawed tumor mince was called 8415B (1st passage)

				Subcutaneous injection			Intraperitoneal injection		
Tumor	Passage	Tissue	Gm. equiv. injected	No.	Cancer (per cent)	Av. ca. age	No.	Cancer (per cent)	Av. ca. age
8415A	1st	Unfrozen	2×10^{-2}	28	86	356	17	71	454
8415A	1st	Unfrozen	10^{-3}	31	94	331	40	88	367
8415A	9th	Unfrozen	5×10^{-2}	0			37	89	335
8415A	9th	Unfrozen	10^{-3}	0			47	94	345
8415A	9th	Unfrozen	10^{-4}	0			45	96	328
8415A	1st	Frozen	2×10^{-2}	31	94	359	27	93	322
8415A	1st	Frozen	10^{-3}	26	81	314	23	100	324
8415B	1st	Unfrozen	2×10^{-2}	26	38	312	21	100	344
8415B	1st	Unfrozen	10^{-3}	28	4	360	28	43	431
8415B	3d	Unfrozen	2×10^{-2}	14	14	395	43	86	346
8415B	3d	Unfrozen	10^{-3}	42	0		27	63	362

MATERIALS AND METHODS

Tumor No. 8415, a spontaneous mammary carcinoma, developed in a breeding female of the AZF_1 (A♀ × Z♂) generation. This tumor was transplanted into $AxZbF_1$ or $ZbAxF_1$ hybrids produced by mating animals of the fostered (without the mammary tumor agent) Ax and Zb lines of the parental strains. The tumor was continued for 31 passages, and the grafts showed progressive growth in all of the 226 inoculated animals.

Fresh tissues from tumors of the first and ninth passages were tested for the mammary tumor agent, and these are referred to as unfrozen tissue of tumor No. 8415A, 1st and 9th passages (Table 1). Transplants of the first passage of tumor No. 8415A were frozen to −79° C. in cellosolve and dry ice, as previously described (5), following the technic outlined by Gye *et al.* After storage for 7 days, the ampules were placed in a water bath maintained at 37° C. until the tissue had thawed.

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Saline was added to the tumor mince, and the suspension was injected subcutaneously into F_1 mice without the agent. Some tumors developed at the site of injection (5); the largest (2.8 gm.) was called 8415B (1st passage) and was assayed for the agent. Tumor 8415B was transplanted for two additional passages when tissue was again tested for the agent.

The test animals were ZBC mice without the mammary tumor agent, 20–24 days of age, of which less than 2 per cent develop spontaneous mammary cancer (3) when maintained as controls. The experimental animals were continued as breeders and given Purina Fox Chow and tap water.

The tissue to be assayed was ground with sand, and distilled water was added to give a 10 per cent suspension (wet weight). This was centrifuged for 10 minutes at approximately 2,500 r.p.m., and the supernatant was recentrifuged for either 10 or 20 minutes at the same speed. The final supernatant was further diluted so that the injection of 1 cc. contained the agent extracted from the amount of tissue given in the table. The route of administration is given for each series.

To compensate for any differential mortality between the various groups of injected mice, only noncancerous animals which lived to the average cancer age have been tabulated. The average cancer and noncancerous ages for all mice were 355 and 603 days, respectively.

RESULTS AND DISCUSSION

The results observed following the administration, either subcutaneous or intraperitoneal, of centrifugates of transplanted mammary tumors, with either frozen or fresh tissue, are presented in Table 1. Details regarding the data will be considered below.

To demonstrate the presence of the mammary tumor agent in either normal or cancerous tissues, cell-free extracts of these tissues are injected into young animals of strains which are susceptible to spontaneous mammary cancer, but which do not possess the agent. If the extract contains the agent, the incidence and time of appearance of mammary cancer in the test animals will be found to be comparable, in general, to those observed in some high cancer stocks.

The mammary tumor agent of mice (1) has been found to have the properties of an infectious agent or virus and will remain active following filtration (3), lyophilization (2), and desiccation (3, 10–13).

The conclusion of Gye *et al.* (16–17) and Mann (20, 21, 24) that tumor cells, including mammary carcinoma, would not survive freezing to low temperatures is contrary to the results obtained by many workers previous to the date of their first publication and to the many reports that have appeared since that time (5, 7–9, 18, 19, 25–29).

Passey *et al.* (26) demonstrated the presence of viable cells in lyophilized tumor material, where active growth was obtained in tissue culture, provided the frozen tumor was not desiccated for longer than 75 minutes (approximately the length of time employed by Gye).

It also has been noted that the injection of the suspended thawed tumor mince would produce tumors at the site of injection only in mice of stocks or their hybrids which were susceptible to grafts of the fresh tumor being used. These tumors would behave, upon further transplantation, like the original tumor (5, 9), showing that they did not develop from the cells of the injected host. The mammary tumor agent, on the other hand, will induce tumors in mice of other strains (see 3, 4) or, as stated by Gye (15), in mice with a susceptibility different from that of the strain from which the agent was obtained.

By using a transplanted mammary tumor, No. 8415A, which developed in an AZF_1 hybrid female, observations have been obtained on the activity of the mammary tumor agent in fresh and frozen tumors of the same passage.

When unfrozen tumors of the first passage were assayed for the agent, higher incidences and earlier cancer ages were observed in the mice which received the extracts subcutaneously than in those which were given the same fractions intraperitoneally. After the tumor had been transplanted for nine passages, transplants were tested, but the mice served as controls for another study, and only intraperitoneal injections were made. The results were comparable to those obtained for the first-passage tumors, regardless of the route of injection, showing that the tumor-producing activity of the agent had not decreased during the interval (Table 1). Transplants of the twelfth and 31st passages have been assayed, and preliminary data demonstrate the survival of the agent in both series.

Transplants of the first passage of tumor 8415A were frozen in cellosolve and dry ice at −79° C., stored for 7 days, thawed at 37° C., extracted in the usual way, and the final supernatant injected into agent-free young mice. The extracts were as active as those of the unfrozen tissue of the same passage, and the tumors appeared as early in mice injected intraperitoneally as subcutaneously. No variation was seen in the age of the mice at the time of appearance of the first tumors in the four groups, the range being from 193 to 206 days after birth.

Following the subcutaneous injection of the thawed tumor suspension of tumor 8415A, frozen for 7 days, the largest tumor to develop was called 8415B, to distinguish it from transplants descended from grafts of the unfrozen tissue of 8415A. The cell-free centrifugates of tumor 8415B

showed a lower tumor-producing activity than did extracts of tumor 8415A, except when the mice received a dose of 2×10^{-2} gm. equivalents of the material intraperitoneally. The same fraction, administered subcutaneously, gave a low incidence. When the extract of 8415B was diluted 1000-fold, fewer mice developed tumors, regardless of the route of injection. In this series, the first groups to be injected were the mice which received the fractions subcutaneously, and litter-mate controls were employed for all groups. The unfrozen tissue of the third passage of tumor 8415B showed further loss of activity following subcutaneous administration but remained relatively the same for intraperitoneal injection of the extracts.

In all groups tested with extracts of tumor 8415B, which appeared after the injection of the thawed suspension of tumor 8415A, the incidence in the experimental animals was dependent not only upon the amount of the agent to be injected but also the site of inoculation. This was not the case with tumor 8415A, when unfrozen tissue of the first and ninth passages and frozen tissue of transplants of the first passage were used.

Considering all groups, regardless of the tissue used or the concentration of the extracts, the average cancer age for mice which received subcutaneous injections was 347 days, as compared to 360 days for those which obtained the extracts intraperitoneally; the average noncancer ages for the respective groups were 664 and 543 days. The average noncancer age for the mice which received extracts of tumor 8415B subcutaneously was 654 days. These mice were continued as breeders, and the average number of litters born to each female was 5.9.

It is obvious that, at this time, no definite explanation may be suggested to account for the results obtained following the injection of the extracts of the tumor, 8415B, which were descended from the viable cells present in the thawed tumor mince. Further studies are contemplated on the problem.

From these studies, it is evident that the mammary tumor agent, an infectious agent or virus, does not become "active" following freezing and induce mammary cancer within a few days or weeks, as claimed by Mann (20, 21, 24). After the injection of cell-free supernatants, the first tumors appeared as early in mice which received extracts of the unfrozen tumors as when frozen tissue was used. Also, the results showed that the agent did not have to come in contact with tubules of the normal gland to produce mammary cancer, since, in most series, intraperitoneal injection was more effective than subcutaneous administration of the same fraction.

SUMMARY

No difference was observed in the tumor-producing activity of the mammary tumor agent in cell-free supernatants of frozen and unfrozen transplanted mammary carcinoma, when tumors of the same passage were used as the source material.

There was no decrease in the activity of the agent derived from fresh tissue after the tumor had been transplanted for nine passages.

When extracts of frozen tumors of the first passage were used, no variation was seen either in the incidence of mammary cancer or the average cancer age in mice injected intraperitoneally or subcutaneously. The first tumor appeared at 193 days.

Tumors appeared at the injection site of the thawed tumor mince. Extracts of these tumors, and their transplants, gave fewer tumors in the test animals when injected subcutaneously than intraperitoneally.

From these studies, no evidence was obtained to suggest that the mammary tumor agent is activated by freezing.

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The Metabolism of Benzidine in the Rat

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The establishment of the carcinogenic activity of benzidine by Spitz, Maguigan, and Dobriner (9) necessitates a more complete understanding of the mechanism of benzidine metabolism in the animal body. Little investigation has so far been carried out. Goldblatt (5) has suggested that acetylbenzidine is excreted in addition to the base, and the work of Weber and Heideprim (10) indicates that benzidine is excreted partly unchanged and partly as what they describe as an ethereal sulphate and glucuronide. The formation of these conjugates indicates that benzidine may be oxidized *in vivo*. Adler (1) has demonstrated a dihydroxy derivative of unknown structure in the urine of rabbits fed benzidine, and Baker (2) has demonstrated that oxidation may take place to give 3,3′-dihydroxybenzidine.

In recent investigations on synthetic 3,3′-dihydroxybenzidine, Baker (3) noted a marked similarity between results of tumor formation in animals fed this compound and those fed 2-acetylaminofluorene (4, 13). This suggested to him that the metabolism of benzidine, 2-aminofluorene, and 2-acetylaminofluorene, respectively, might follow a similar, if not parallel, course in the rat.

The present study, part of a series of studies on the metabolism and degradation of benzidine, was undertaken to determine the distribution of benzidine in the rat, the extent of conjugation of the aromatic amino groups, and the rate of recovery of benzidine from tissue and other biological materials.

MATERIALS AND METHODS

Animals.—Male Slonaker rats, bred and maintained in this laboratory, were used. At the start of the experiment they weighed 200–250 gm.

Diet.—Prior to the experiments, the animals were maintained on Ministry of Food rat cake 41,[1] supplemented with green vegetables and Adexolin.[2]

Preparation and administration of chemicals.—Benzidine, m. p. 128° C. (corr.), was prepared by a zinc reduction of nitrobenzene. This was purified by recrystallization and fractional sublimation.

The chemical was dissolved in 1–2 ml. of warm propylene glycol for intraperitoneal injections, and all animals received 100 mg of the chemical/kg of body weight. The toleration of this dose had been previously established. Following injection, the animals were placed in metabolism cages; water was allowed ad libitum, but food was withheld during the experimental period.

Preparation and extraction of tissues.—The method of Gutman, Kiely, and Klein (6), a modification of that of Westfall (11), and the method of Morris and Westfall (8) were used in the preparation and extraction of tissues and biological material throughout this series. These consisted of homogenizing pooled samples of tissue and extracting with acetone. Following evaporation of the solvent, the residues were washed in a measured amount of hot glacial acetic acid, and the wash liquid was made up to 10 ml. with distilled water. After cooling and filtering, the estimation of diazotizable amino groups was carried out.

Determination of benzidine.—The diazotization method of Westfall (11), as modified by Gutman, Kiely, and Klein (6), in which tissue extracts were diazotized with 0.029 M sodium nitrite and coupled with 0.031 M sodium 2-naphthol-3,6-disulfonate in 10 M aqueous ammonium hydroxide, was used for estimating the presence of benzidine. The optical density (log K) of the resultant dye solution was immediately determined at a wave length of 545 mμ (Chart 1), using a Spekker photoelectric absorptiometer. The readings obtained were expressed in μg. of benzidine by reference to a previously established calibration curve which obeyed Beer's law within a range of 0–50 μg.

The values thus obtained are expressed as "free" diazotizable material. The amino groups

[1] Ministry of Food rat cake 41 consists of whole meal flour, 46 per cent; oats, 40 per cent; fish meal, 8 per cent; dried skimmed milk, 3 per cent; 1 per cent each of dried yeast, cod liver oil, and salt.

[2] Adexolin, a proprietary vitamin concentration of Vitamin A and D in oil; 1 ml. contains 12,000 units Vitamin A and 2,000 units Vitamin D.

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determined after acid hydrolysis according to the method of Westfall and Morris (12) are referred to as "total" diazotizable material. When benzidine was added to tissue homogenates, 80—100 per cent could be recovered by this method.

RESULTS AND CONCLUSIONS

From the results listed in Table 1, it will be noted that the amounts of diazotizable materials, 4 and 12 hours after administration of benzidine, varied considerably from tissue to tissue. After 4 hours, the concentration of total diazotizable aromatic amino groups in rat tissue ranged from 310 μg/gm in stomach to 11 μg/gm in red cells; and after 12 hours, from 135 μg/gm in small intestines to 5 μg/gm in red cells.

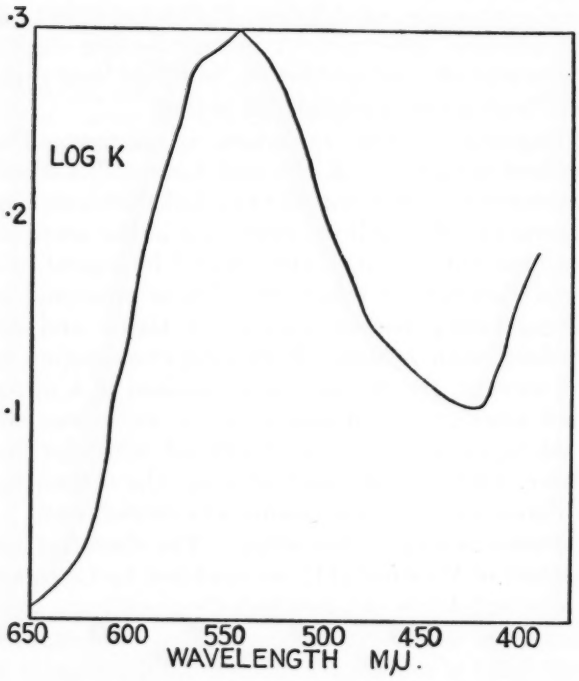

CHART 1.—Absorption spectrum of 4,4′-bis-(1-azo-2-naphthol-3,6-disulfonic acid)-biphenyl in 0.06 M acetic acid. Concentration of compound was 0.01 M; cell thickness was 10 mm.

Although all the tissues analyzed contained "free" diazotizable material, additional aromatic amino groups became available after acid hydrolysis. This conjugation of benzidine may be of importance in considering whether acetylation is involved in benzidine metabolism, as has been suspected (5); that the rat is able both to acetylate and to deacetylate aromatic amino groups has been shown by Morris *et al.* (7) working with 2-acetylaminofluorene. One may presume that a similar mechanism is involved here and that acetylbenzidine is a product of benzidine metabolism.

The percental recovery and distribution of benzidine, measured as diazotizable amino groups, are also shown in Table 1. After 4 hours, 93 per cent of the administered amino groups were accounted for after acid hydrolysis, diazotization, and coupling with R-salt. Approximately 24 per cent were in the conjugated form; 29 per cent of the total amino groups recovered were found in the carcass, 25 per cent in the small intestines, and approximately 5 per cent in the liver. Only 0.5 per cent of the recovered amino groups were found in the urine, compared to over 15 per cent in the contents of the gastrointestinal tract.

Twelve hours after administration of the chemical, 68 per cent of the administered amino groups were recovered, 49 per cent being in the conjugated form. The general decrease in the measurable diazotizable aromatic amino groups in tissue after 12 hours is similar to that found by Gutman *et al.* (6) working with 2-aminofluorene and by Westfall and Morris (12) working with 2-acetylaminofluorene, although with benzidine the extent of conjugation is very much greater. In most of the tissues there was a decrease in the recovery of injected amino groups at 12 hours, the largest decreases occurring in the stomach contents and carcass.

That a significant level of diazotizable material was maintained in the liver for at least a 12-hour period is of interest, since benzidine, like 2-aminofluorene and 2-acetylaminofluorene, induces tumors in this organ.

The amount of diazotizable amino groups found in the urine after 12 hours was 5 times and that in the contents of the gastrointestinal tract about twice as great as at 4 hours. The marked increase in the urinary level indicates that benzidine is excreted by the urinary tract, as are 2-aminofluorene and 2-acetylaminofluorene. In addition, there is evidence that benzidine is also excreted by the intestinal tract to a great extent, since a portion of the products of metabolism are excreted by the bile to the stomach and intestinal tract.

After 4 hours, 93 per cent of the injected amino groups were recovered, after acid hydrolysis, diazotization, and coupling with R-salt, but only 68 per cent of the injected amino groups were recovered after 12 hours. The chemical reaction by which the amino groups of benzidine were rendered nondiazotizable after 12 hours is not known, but it is suspected that this loss may be due to *in vivo* oxidation. Experiments designed to isolate the products of *in vivo* oxidation and their identification are under way.

SUMMARY

The distribution of benzidine, expressed as diazotizable amino groups following a single intraperitoneal injection, has been studied in the rat.

The aromatic amino groups were present in both the "free" and conjugated form, the latter amounting to 24 or 49 per cent of the dose after 4 or 12 hours.

By 12 hours, a portion of the injected amino groups are rendered nondiazotizable, since only 68 per cent could be accounted for at this time, as compared to 93 per cent 4 hours after injection.

It is suggested, in the light of the similarity of benzidine and 2-aminofluorene metabolism, that this difference in recovery may be due, in part, to *in vivo* oxidation.

TABLE 1

THE CONCENTRATION AND DISTRIBUTION OF FREE AND TOTAL DIAZOTIZABLE MATERIAL IN RAT TISSUES
Single Intraperitoneal Injection of 100 Mg Benzidine/Kg of Body Weight

	(4 hours)*				(12 hours)*			
	Free		Total		Free		Total	
Tissue	μg/gm	Per cent of total	μg/gm	Per cent of total	μg/gm	Per cent of total	μg/gm	Per cent of total
Whole blood†	16.2		35.0		1.0‡		17.5	
Plasma†	32.5	0.3	70.0	0.7	1.0‡	<0.1	15.0	0.2
Blood cells†	8.7	1.1	11.2	0.1	3.8	<0.1	5.0	0.1
Liver	16.2	1.7	45.0	4.8	11.3	1.6	25.0	3.5
Spleen	21.2	0.2	46.2	0.5	6.3	0.1	17.5	0.2
Kidney	21.2	0.2	46.2	0.5	2.5	0.1	11.2	0.2
Stomach	275.8	8.3	310.0	9.3	20.0	0.6	80.0	2.4
Stomach contents	310.0	15.6	315.0	15.8	54.0	2.7	85.0	4.3
Small intestines	81.2	13.0	157.5	25.2	26.3	2.2	135.0	11.5
Small intestine contents#					134.0	6.7	560.0	28.0
Caecum	2.5	0.1	21.2	0.6	13.8	0.3	75.0	1.9
Caecum contents	2.0	0.1	11.4	0.6	0.1	<0.1	15.0	0.8
Colon	3.7	0.2	21.2	1.6	1.0	0.1	25.0	1.3
Urine	5.4	0.3	10.2	0.5	28.0	1.4	50.0	2.5
Miscellaneous§	1.7	0.3	21.2	3.8	1.0	0.2	11.2	2.0
Carcass	81.2	28.8	82.5	29.3	8.8	3.2	36.2	13.5
Total		69.2		93.3		19.20		72.4

* The analyses were performed on the pooled samples of four rats.
† These figures calculated from 2 ml. of pooled blood.
‡ Less than 1.00 μg.
# Not estimated for 4 hours.
§ The miscellaneous sample consisted of brain, pancreas, diaphragm, bladder, gonads, esophagus, heart, thymus, lungs, and adrenals.

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Effect of Limited Food Intake on Survival of Mice Bearing Spontaneous Mammary Carcinoma and on the Incidence of Lung Metastases*†

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It has long been known that the growth of neoplasms in animals can be retarded by the reduction of food intake. This applies to spontaneous and deliberately induced tumors as well as to those developing from transplants. Both simple underfeeding and restriction of carbohydrate only (caloric restriction) are effective (11). The influence of food restriction on the longevity of the tumor-bearing host, however, has received little attention. Sugiura and Benedict demonstrated that underfeeding, instituted after surgical removal of spontaneous mammary tumors, delayed recurrence of the neoplasms and extended the life of the mice (8). Flory and co-workers investigated the effect of underfeeding on mice which had been inoculated with leukemic cells 2 days previously (3). With two strains of transplantable leukemia survival time was increased, whereas with two other strains no influence was observed. Neither study answers the question whether underfeeding or caloric restriction—without surgery or other treatment—alters the life span of animals with established, growing tumors. This was a principal objective of the present study with mice bearing spontaneous mammary carcinoma. Furthermore, since mice of the strain utilized often develop more than one breast tumor and exhibit metastases to the lungs, it was possible to garner information relevant to the influence of underfeeding on these events.

MATERIALS AND METHODS

Employing the following general conditions, three experiments were performed consecutively over a period of several years. A large group of C3H female mice, born within a 2-week span, were housed in sets of five per cage; they were offered Purina Laboratory Chow, an adequate commercial diet, and water ad libitum. At 2-week intervals the mice were weighed and inspected for neoplasms. The animals in which tumors had emerged were paired according to their body weights and the location and dimensions of the neoplasms; those which could not be paired were discarded—resulting in rejection of approximately half the original stock. The selected animals were housed individually and entered into the experiment. One animal of each pair was assigned to the "full-fed" group, and fed a daily ration of 13 Calories; the other, placed in the "restricted" group, received 7.4 Calories.

In Experiments 1 and 2, both full-fed and restricted mice were fed a diet of the following composition: Purina Fox Chow meal, 50 per cent; skimmed milk powder, 25 per cent; cornstarch, 20 per cent; brewers yeast, 3 per cent; and partially hydrogenated cottonseed oil, 2 per cent. Full-fed mice were given and consumed 3.6 gm., underfed mice 2.0 gm. daily. In Experiment 3, the full-fed rations of 3.6 gm. consisted of Purina meal, 1.2 gm.; milk powder, 0.7 gm.; brewers yeast, 0.1 gm.; and cornstarch, 1.6 gm. The restricted ration contained the same amounts of meal, milk powder, and brewers yeast (i.e., the same amounts of protein, fat, minerals, and vitamins), but the cornstarch was reduced to 0.1 gm. to make a total of 2.1 gm. daily. Thus, the restricted animals of the first two experiments were "underfed," those of the third experiment were "calorie-restricted" (11).

The animals were weighed and inspected at 2-week intervals, and their general condition, including the appearance of new mammary carcinoma, was noted. All neoplasms were measured when the mice were placed in the experiment and at death. In Experiment 2 the growth rates of the tumors were evaluated by semiweekly measurements; in Experiment 3 all mammary tumors of a mouse, and its carcass (exclusive of tumors), were weighed.

The first two experiments were designed to determine mainly the influence of reduced food intake on the life span of the tumor-bearing animal, and, hence, all mice were permitted to survive until death; supplementary observations were made on the occurrence of additional mammary carcinomas and the presence of grossly visible metastases to the lungs. On the other hand, the primary objective of the third experiment was evaluation of the effect of caloric restriction on the occurrence of metastases and additional mammary tumors; consequently, when one animal of a pair succumbed, its mate was sacrificed.

The mammary tumors and lungs of each mouse with lung nodules were examined histologically. In every instance the lung tumors were found to be secondary to the mammary neoplasms. Sections of breast tumors of numerous animals without metastatic lesions also were examined.

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RESULTS

Chronic restriction of food intake had a beneficial effect on the life span[1] of mice bearing mammary carcinoma (Table 1). This is shown in both Experiments 1 and 2, in which the average survival time of the restricted animals was 2–3 weeks longer than that of their full-fed mates. In all three experiments, furthermore, the mouse on the limited regimen outlived its control in approximately two-thirds of the pairs. These findings are statistically significant.

Fewer of the restricted mice developed additional mammary carcinomas or grossly visible metastases to the lungs (Table 2). Moreover, their metastatic lesions were less numerous and smaller in size. These salutary effects occurred in all three experiments. They were more critically evaluated in Experiment 3, in which differences in life span were eliminated by sacrificing the mate when an animal died. In this study, the proportion of calorie-restricted mice with additional mammary neoplasms or metastases was significantly less, statistically, than the corresponding values for the full-fed controls.

The lower frequency of additional mammary tumors and of metastases was not the basis of the prolonged life span of the restricted mice. The

[1] The interval between detection of the tumor and death of the animal is meant in this and all subsequent references to life span, longevity, and survival time.

TABLE 1

INFLUENCE OF RESTRICTED FOOD INTAKE ON SURVIVAL OF MICE BEARING SPONTANEOUS MAMMARY CARCINOMA

Exp.	No. pairs	Survival time* of full-fed mice (days)	Increase in survival time† of restricted mice (days)	Proportion of pairs in which the restricted mouse outlived its full-fed control (per cent)
1	40	83±4.8 (37–145)	+23.8±7.93 (−79 to +162)	65
2	53	74±4.5 (29–178)	+16.2±6.61 (−101 to +190)	69
3	70	82±4.4 (23–140)	‡	63

* Interval from detection of the tumor until death of the animal: mean ± standard error of the mean and, in parentheses, range of individual values.

† Survival time of restricted mouse minus that of its respective full-fed control: mean ± standard error of the mean and, in parentheses, range of differences.

‡ These data were not obtained in Experiment 3—when an animal died, its mate was sacrificed.

TABLE 2

INFLUENCE OF RESTRICTED FOOD INTAKE ON MICE BEARING SPONTANEOUS MAMMARY CARCINOMA: ADDITIONAL MAMMARY NEOPLASMS, LUNG METASTASES, AND TUMOR GROWTH

Exp.	Group	No. mice	Proportion of mice bearing additional mammary tumors (per cent)	Incidence of mice with visible lung metastases (per cent)	Tumor growth*
					Diameters (mm.)†
1	Full-fed	40	50	35	26.0±1.29 (14–36)
	Underfed	40	15	20	21.7±0.67 (12–27)
					Growth rates (mm/day)‡
2	Full-fed	53	51	36	0.35±0.03 (0.10–0.77)
	Underfed	53	42	27	0.23±0.02 (0.04–0.41)
					Weights (gm.)§
3	Full-fed	70	51	23	10.2±0.57 (1–25)
	Calorie-restricted	70	33	6	4.6±0.33 (0.5–13)

* These are means ± standard errors of the means and, in parentheses, the ranges of various measures of tumor growth. In Experiments 1 and 2 the values refer only to those animals with a single tumor; similar figures were obtained when only *initial* tumors of *all* animals were considered. The differences between the full-fed and restricted values are statistically significant.

† Final diameter (average of major and minor axes) of the tumor.

‡ Rate of increase of diameter.

§ Total weight of all mammary neoplasms born by the individual animal.

differences in survival for only those pairs in which both animals bore single neoplasms and no metastases were essentially the same as those given in Table 1 for all animals.

The last column of Table 2 illustrates the influence of limited food intake on the growth of mammary carcinoma. The tumors of the restricted mice, compared to those of the controls, averaged 16 per cent smaller in final diameter (Experiment 1), grew at approximately two-thirds the rate (Experiment 2), and attained less than half the weight (Experiment 3). Furthermore, in over 80 per cent of the pairs these measurements were smaller in the restricted animal.

As should be expected the restricted mice lost more weight than the full-fed. For example, in Experiment 3 the mean initial body weights for both groups was 33 gm.; at death the carcass weight of the full-fed animals averaged 24 gm., the calorie-restricted animals 19 gm.

DISCUSSION

Underfeeding or caloric restriction of mice bearing mammary carcinoma of spontaneous origin increased their life span, decreased the rate of growth of the tumors, hindered the formation of additional neoplasms of the mammae, and decreased the frequency of lung metastases.

Initial criteria and statistical evaluation.—Before proceeding with a discussion of the results, the initial conditions, the criteria used for pairing, and some statistical considerations are reviewed. The initial conditions were those present when the neoplasms were first detected, and the animals paired and entered into the study: the factors of selection were the diameters and sites of the tumors, and body weights and ages of the mice. Construction of the two groups of a study by pairing the animals according to these four criteria —rather than through random distribution—was not only rational but also resulted in increased precision of the comparisons (7).

The actual initial conditions may be summarized as follows: diameters of the carcinomas (average of major and minor axes) ranged from 3 to 15 mm. (mean, 8 mm.); the body weights varied from 26 to 43 gm. (mean, 33 gm.); the tumors were in many sites, all related to the anatomic location of the mammae; and the mice were paired at from 30 to 70 weeks of age.

In selecting the two members of a pair, virtual equality of age of the animals and of location of the tumors was achieved. Equating them with respect to body weight and dimensions of the tumors presented more difficulties. In fact, relatively lenient limits were employed: the diameters of tumors accepted for a pair differed by as much as 5 mm., although in 80 per cent of the pairs the difference was less than 1.5 mm. Similarly, body weights differed occasionally by as much as 10 gm.; in about 80 per cent of the pairs the difference was less than 3 gm. For both measurements the means of the differences were negligible. Analysis of our material demonstrated that the divergence in initial body weight or in initial tumor diameter of a pair was not a factor in the effect of food restriction on survival time, growth of the tumor, occurrence of additional mammary neoplasms, and frequency of metastases.

Statistical evaluation of the results was complicated by the fact that, in the main, the data did not follow normal distributions. It was therefore necessary to transform the data in order to permit application of the usual tests of significance (2). In Experiments 1 and 2, for example, the survival times of the mice, as well as the differences between the two members of each pair, were markedly skew in distribution. However, the logarithms of the ratios of the survival times of the restricted mice to those of their full-fed mate were normally distributed with mean values of 0.089 ± 0.039 and 0.072 ± 0.029 for Experiments 1 and 2, respectively. Both means are statistically greater than zero and indicate that, on the average, the underfed mice lived 20 per cent longer—from the time they were entered into the study—than the full-fed controls.

Growth of tumors.—The present results agree with the generally accepted fact that underfeeding or caloric restriction hinders the growth of tumors (11). This is shown not only in Experiment 3, where the underfed and full-fed mice had equivalent survival times, but even in Experiments 1 and 2, where the tumors of the underfed mice had about 20 per cent more time for growing. In all three studies the findings related to growth proper, thus differing from some reported experiments with implanted tumors where the results were compounded from effects on establishment and on growth.

Multiple mammary carcinomas.—Early studies by Sugiura and Benedict (8) suggest that, in mice from which initial mammary carcinomas had been excised, the development of new neoplasms was significantly inhibited by underfeeding. Morris and Robertson reported that additional mammary tumors formed much less frequently in tumor-bearing mice placed on a riboflavin-deficient diet (5). Inasmuch as riboflavin deficiency leads to reduced food intake and loss of body weight, their results are probably related to those of the present investigation in which underfeeding

or caloric restriction alone hindered the appearance of additional mammary tumors.

These findings are added proof that the genesis of neoplasms can be inhibited by caloric restriction instituted shortly before tumors are expected to appear. To this extent they support the view that restricted food intake exerts its main inhibitory effect on tumor genesis during the developmental, rather than the initiatory, stage of carcinogenesis (10).

Metastases.—To our knowledge, a decrease in frequency of metastases as a result of limited food consumption has not been previously demonstrated. Metastases may be considered to be the consequence of a series of events: invasion of blood or lymph vessels by the primary neoplasm, formation of emboli, survival and establishment of the emboli in the tissue of lodgement, and, finally, growth. Caloric restriction or underfeeding might very well act on all these stages. Little is known about the influence of nutrition on invasion and embolism, but its action on the establishment and growth of a metastatic embolus may be analogous to that on implants—particularly autotransplants. Underfeeding nearly always retards the establishment, and sometimes the growth, of transplants. Perhaps, as suggested by Rous (6), it limits "the host's ability to form a connective tissue scaffolding and vascularization necessary for their (the implants') support." The same idea, invoked for the action of food restriction on the establishment and growth of metastases, may explain their decreased frequency in the restricted mice.

Longevity.—Probably, the prolonged survival of the tumor-bearing underfed mouse was a resultant of two primary influences: (*a*) on the animal; (*b*) on the neoplasm.

It has been demonstrated conclusively that caloric restriction prolongs life (1, 4, 9). This beneficial effect on the animal presumably occurs even though a tumor is present and growing. However, the neoplasm, too, plays a part in determining the life span of the host: Generally, the more slowly a tumor grows, the longer is the survival time. In Experiment 2, the co-efficient of linear correlation between growth rates of the carcinomas and longevity was -0.66 for the full-fed controls and -0.54 for the underfed mice. If a cause-and-effect relationship is assumed, these values can be broadly interpreted as implying that a little more than half the factors that controlled survival arose from the rapidity of tumor growth. Probably, any procedure which retards the growth of the neoplasm—*and in itself has no untoward effect on the host*—is likely to prolong the life span. Moderate underfeeding or caloric restriction is in this category. In accordance with this viewpoint, the difference in life span between members of a pair was compared to the difference in growth rate of their tumors: there was a statistically significant linear correlation (-0.40), denoting that, to some extent, the greater the inhibition of tumor growth through underfeeding, the greater the increase in survival time.

The longer *average* life span of the restricted animals was not attributable to the reduced incidence of additional neoplasms and metastases. Furthermore, there was no association between which animal of a pair died first and which developed metastases or multiple mammary tumors. However, these events did curtail the life of some *individual* animals. Actually, in about 10 per cent of the full-fed mice and a few of the underfed, death was due to virtually complete replacement of the lungs by metastatic nodules. However, these were mainly animals of longer-than-average survival time, and the over-all mean values for the differences between the full-fed and restricted mice were not modified by excluding them from the computations.

Conclusions.—In this study the salutary action of underfeeding or caloric restriction on longevity and the frequency of metastases has been demonstrated for a single tumor type—one that grows slowly, permits a relatively long survival, and metastasizes with only moderate frequency and almost exclusively to the lungs. It is not at all probable that effects of the same *degree* would be observed with rapidly growing neoplasms and with those that metastasize extensively. Nevertheless, since caloric restriction has been shown to repress strikingly the genesis and growth of so many different kinds of neoplasms, it is not unlikely that the beneficial influence upon longevity and metastases also has generality. We believe that, with many neoplasms, the *early* institution of caloric restriction might result in retarded growth and spread of the tumor, and in prolongation of the life of the host. This is not suggested as a practical measure for cancer in man, since it probably in no way compares with the results and potentialities of surgery and irradiation.

SUMMARY

The influence of restricted food intake on the survival of mice with spontaneous mammary carcinoma was investigated in three experiments. Strain C3H mice with small single tumors were paired according to age and body weight, as well as size and location of the neoplasms. One of each pair was full-fed (13 Calories daily), the other restricted (7.4 Calories). Limitation of food intake

was achieved by either proportionate reduction of all dietary components (underfeeding) or by decrease of carbohydrate only (caloric restriction). A total of 163 pairs of mice was used in the study.

The average survival time of the tumor-bearing mice on the low-calorie rations was about 20 per cent longer than that of the full-fed controls. Furthermore, in two-thirds of the pairs the restricted mouse outlived its respective mate. The limitation of food intake also resulted in a decreased rate of growth of the tumors, reduced incidence of additional mammary carcinomas, and lower frequency of grossly visible metastases to the lungs.

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Substrate Utilization by Ehrlich Mouse Ascites Carcinoma Cells*

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The rapid rate of growth and multiplication of the Ehrlich mouse ascites carcinoma cells (6) indicates the need for large amounts of nutrients for the production of protoplasm and the acquiring of energy for those synthetic processes. Recently, Christensen and Riggs (2) and Christensen, Riggs, Fischer, and Palatine (3) have reported the striking ability of this tumor cell to concentrate amino acids, and Klein (4) has studied nucleic acid synthesis. Kun, Talalay, and Williams-Ashman (5) have considered a number of aspects of the energy-yielding metabolic processes of this cell.

In this study we are reporting data on (*a*) various methods of preparing and handling the ascites carcinoma cells and the use of a washed cell system for metabolic studies, (*b*) the substantially increased rate of oxidation over the endogenous level with added lactate, pyruvate, oxalacetate, malate, succinate, α-ketoglutarate, citrate, glutamate, aspartate, and alanine, and (*c*) the rapid rate of glycolysis and the markedly slower rate of oxidation in the presence of glucose.

MATERIALS AND METHODS

Ascites infection.—The Ehrlich mouse ascites carcinoma cells, a variant of the Ehrlich breast carcinoma of Lowenthal and Jahn (7), which we used in this investigation, were obtained from Drs. Halvor N. Christensen and Thomas R. Riggs of the Department of Biochemistry and Nutrition of Tufts College Medical School. The tumor cells were propagated in male and female C57BL mice, 5–8 weeks of age, by intraperitoneal injection of 0.2 ml. of the ascites tumor material (15–25 million tumor cells). Within 6–9 days following the inoculation, 6–10 ml. of ascites tumor material was obtained. The mice were sacrificed by a sharp blow on the head and decapitation. The peritoneal fluid was removed by means of a medicine dropper through a small opening in the peritoneal wall. This liquid tumor material usually had a packed cell volume of 20–30 per cent and a cell count of about 1.2×10^5/c. mm. There were also present 5–10 per cent by volume of leukocytes and a highly variable amount of red cells (0.2–15 per cent by volume).

The tumor material was prepared for study in several ways (Table 1): (*a*) the whole fluid material as taken from the mouse; (*b*) 2× washed ascites tumor cells; and (*c*) 4× washed tumor cells. Washing of the cells was carried out either at room temperature or at +5° C. The washing was effected by suspending the ascites material in 5 volumes of isotonic phosphate-Locke's solution (8) (pH 7.3, freezing point depression −0.54° C.), centrifuging at 350 × *g* (1,200 r.p.m. in the No. 2 International Centrifuge) for 5 minutes, and discarding the supernatant. The process was repeated once for the 2× washed cells and thrice for the 4× washed cells. The tumor cells were then suspended in iso-

TABLE 1

OXYGEN CONSUMPTION BY EHRLICH MOUSE ASCITES CARCINOMA CELLS

(Washed 1 ×, 2 ×, and 4 × at room temperature and +5° C., with and without added DL-sodium lactate)

Respiratory period (hr.)	c mm oxygen/hr/0.04 ml tumor cells					
	1× washed		2× washed		4× washed	
	no lactate	17 mM lactate	no lactate	17 mM lactate	no lactate	17 mM lactate
			(5° C.)			
1	93	96	93	98	83	99
2	81	81	79	91	71	90
3	67	89	64	87	54	86
4	46	75	40	79	32	85
			(Room temperature)			
1	85	86	81	91	79	91
2	80	88	74	94	72	97
3	54	65	47	76	44	80
4	46	76	46	78	46	78

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tonic phosphate-Locke's solution to give a packed cell volume of approximately 20 per cent.

When the volume of red cells made up more than 3 or 4 per cent of the material, the erythrocytes were removed by means of a fourfold washing procedure similar to that described above, except that the centrifugation was carried out at about $50 \times g$ (400 r.p.m. in the No. 2 International centrifuge). The fluid phases which contained the red cells were discarded. This process always yielded excellent preparations, grayish in color and containing 75 per cent of the original tumor cells but with only a small portion of the red cells remaining. As indicated by the data in Table 1, such a centrifuging-washing procedure is not detrimental to the oxidative metabolism of the cells. Also,

TABLE 2

THE EFFECT OF SUCCESSIVE WASHINGS ON THE DRY WEIGHT AND RED BLOOD CELL CONTENT OF EHRLICH MOUSE ASCITES CARCINOMA CELLS

Days after inoculation of mice with tumor cells	Dry weights (mg)/0.04 ml. of tumor cells			No. red cells ($\times 10^6$/0.04 ml of tumor cells)*		
	nonwashed	2×	4×	nonwashed	2×	4×
6	7.2	7.5	7.2			
9	7.6	7.8	7.8	11.2	2.8	0.2
12	6.6†	7.8	7.5			
12	5.4†	7.1	7.0			

* 0.04 ml. of cells contains about 17×10^6 tumor cells.

† These two tumor materials were bloody and, before washing, contained 10–15 per cent by volume of red cells.

since these preparations have a low and fairly constant number of red cells, this procedure is recommended for use in all metabolic studies.

With each different tumor cell preparation, the volume of isotonic suspending medium was adjusted to give a cell volume of about 20 per cent. The packed cell volumes were determined precisely by placing approximately 0.2 ml. of the material in an open-end capillary tube (1.5 mm. bore and about 15 cm. long), sealing one end with putty or plastic clay, placing a heavy rubber band lengthwise around the tube, and centrifuging for 30 minutes at $2{,}000 \times g$. The total material and cells were measured with a millimeter rule, and the cell volume was calculated. In some instances dry weight determinations were made on tumor material from mice infected 6, 9, or 12 days previously. In a number of experiments tumor cell counts were made in the same manner as for red cell counts. Although there was not good correlation between cell counts and cell volumes, there was excellent correlation between cell volumes and dry weights (Table 2). Thus, cell volumes were used routinely for all the calculations.

Measurement of respiration and aerobic glycolysis.—The conventional Warburg technic was utilized for the oxidative metabolic studies with the exception that small size (7-ml.) Warburg vessels were employed. The main compartment contained 0.2 ml. of the tumor cell suspension (approximately 0.04 ml. packed cells or 17×10^6 cells), 0.1 ml. of substrate, and 0.6 ml. of isotonic phosphate-Locke's suspending medium. A 15×20-mm. accordion of Whatman No. 40 filter paper and 0.1 ml. of 15 per cent potassium hydroxide were placed in the center well. Measurements of aerobic glycolysis were made employing both the above type of set-up and 25-ml. Erlenmeyer flasks, without means for carbon dioxide absorption. Similar results were obtained with both procedures. All measurements were made at 38°C. following ten minute periods of equilibration.

Glucose and lactate determinations.—Glucose was measured by the Somogyi procedure employing the Nelson arsenomolybdate color reagent (9). Lactate was determined by the Barker-Summerson *p*-hydroxydiphenyl method (1). Glucose disappearance was calculated as the difference between the amount of glucose present at the beginning and at the end of the time interval. Lactate utilization was calculated as the sum of the initial lactate and the glucose which disappeared, minus the lactate present at the end of the time interval. This calculation makes the assumption that the glucose which disappeared was converted to lactate.

Tonicity and pH measurements.—The tonicities of the phosphate-Locke's suspending medium and the modifications of it were determined by freezing point depression measurements made with a Beckmann differential thermometer. The pH measurements on the suspending media and the respiratory systems at the end of the incubations were made by means of the Beckman, Model G, glass electrode pH meter.

RESULTS

The effects of (*a*) total ionic strength, (*b*) inorganic composition of the isotonic suspending medium, and (*c*) pH on the rate of oxidative metabolism of the Ehrlich mouse ascites carcinoma cells were investigated. It was determined that wide variations in the ionic strength had little influence on the respiration. In fact, only when changes of 50 per cent or more were made was there any appreciable effect on oxygen consumption.

Similarly, the potassium content of the medium had little effect until a concentration above 55 mM was present in the system, at which point there was a progressive inhibition of respiration. At 110

mM potassium, an inhibition of 50 per cent was obtained. In these experiments the ionic strength was kept constant by the removal of sodium chloride equivalent to the potassium chloride added. The presence or absence of Mg^{++} had little or no influence on respiration; however, the removal of Mn^{++} from the medium enhanced oxidation about 10 per cent. Likewise, wide variations (14–65 mM) in the phosphate content of the suspending medium at a constant pH had very little effect on respiration. At 85 mM phosphate there was about a 25 per cent decrease in oxidation.

As would be expected, the hydrogen ion concentration of the system is critical to the oxidative

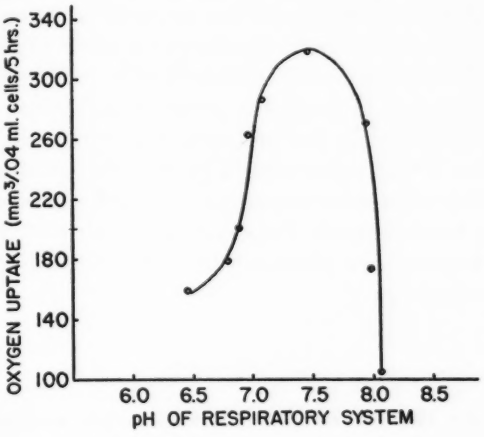

Chart 1.—Influence of the pH of the suspending medium on oxygen consumption by Ehrlich mouse ascites carcinoma cells.

enzyme systems of the tumor cells. This may be seen from Chart 1, which gives the oxygen consumption values for 5-hour respiratory periods. Differences in pH were attained by changing the ratio of the mono- and dibasic phosphates, keeping the total phosphate constant. In these experiments two distinct series of curves were obtained when oxygen uptake was plotted against time (curves not shown): (*a*) at pH values below 7.4 the plots were linear, and the slopes decreased as the pH diminished; (*b*) at pH values higher than 7.4 the plots had a negative curvature beginning shortly after 0 time. The negative curvature increased with increasing pH values, probably owing to greater enzyme destruction at higher pH.

The data in Table 1 show that the washing and centrifuging procedure lowered the concentration of substrates in the tumor cells to a point where oxygen consumption fell off markedly. This effect progressively increased from the 1st hour through the remainder of the 4-hour respiratory period. Although room temperature appeared to be satisfactory for the washing, the procedure was routinely carried out at +5° C. or at room temperature with ice-cold isotonic suspending medium and washing 4 times. That there was no damage to the oxidative systems of the cells and that a lowering of the substrates had been effected was demonstrated by the stimulation of the oxidative metabolism with added lactate (Chart 2 and Table 3).

In addition to the stimulation of oxygen consumption in the washed cell system by lactate, the oxygen uptake of tumor cells was enhanced by pyruvate, oxalacetate, malate, succinate, α-ketoglutarate, citrate, glutamate, aspartate, and alanine (Table 3).

As shown in Table 4, lactate is consumed at a rate of 0.13–0.27 mg/0.04 ml of cells/hour. In these experiments lactate utilization is twofold greater when glucose is the source of oxidative substrate than when lactate per se is added. The tumor cells (0.04 ml.) utilize glucose in the presence of air at a rate of about 0.96 mg/hour. This is an aerobic glycolytic rate some 25 times faster than that of red blood cells.

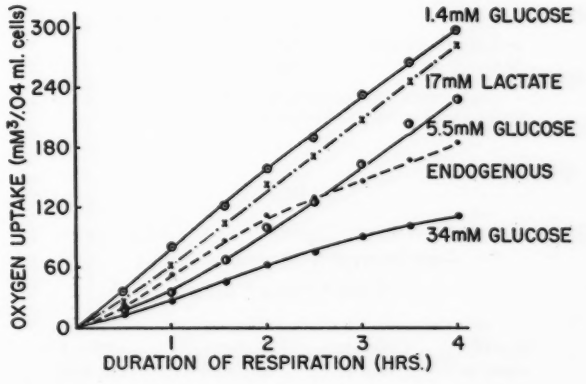

Chart 2.—The influence of added lactate and glucose on the rate of oxygen consumption by Ehrlich mouse ascites carcinoma cells.

One of the most interesting effects of glucose is its ability to stimulate oxidative metabolism at very low levels and to inhibit oxidation at higher levels (Chart 2). Although pH is a critical factor for respiration (Chart 1) the inhibitory effect is not one of excess acidity, since the inhibition is immediate with the addition of glucose; and, as noted in Chart 2, the inhibitory effect produced with 5.5 mM glucose is released after 1 hour, at which time the glucose has all disappeared, and most of the carbon atoms are present as lactate. Furthermore, the addition of alkali to the system after 30 minutes, in a quantity adequate to neutralize the acid formed, did not release the inhibition. The effects of various intermediate metabolites, coenzymes,

and metal activators are being investigated in an effort to determine the reason for this very striking influence.

DISCUSSION

The Ehrlich mouse ascites carcinoma cell is a valuable model system for studying the biological characteristics of abnormally growing and nondifferentiating cells. These cells are of particular value, because they can be rapidly obtained in relatively large quantities and can be freed almost completely of red blood cells. The presence of a small percentage of leukocytes is still an unsolved difficulty. The facts that they can be freely washed and centrifuged and that their numbers can be quantitated by counting, by dry weight determination, and by cell volume measurement are added virtues. These qualities have made it possible to reduce the exogenous substrates of the cells and to show a stimulatory effect on oxidative metabolism with added substrates.

TABLE 3

STIMULATION OF THE ENDOGENOUS OXYGEN CONSUMPTION OF EHRLICH MOUSE ASCITES CARCINOMA CELLS WITH ADDED SUBSTRATES

Substrate	Concentration (mM/L)	Per cent stimulation as measured in 3d hour of respiration
L-Glutamate	10	111 (4)*
DL-Lactate	10	98 (6)
Pyruvate	10	83 (4)
Oxalacetate	10	58 (2)
L-Aspartate	10	50 (4)
DL-Alanine	10	50 (4)
Succinate	10	30 (2)
Citrate	10	27 (2)
α-Ketoglutarate	10	25 (2)
Malate	10	21 (2)

* The figures in parentheses are the numbers of experiments. The values obtained are averages.

TABLE 4

UTILIZATION OF GLUCOSE AND DL-LACTATE BY EHRLICH MOUSE ASCITES CARCINOMA CELLS

Substrate added to washed cells	Concentration of Substrate (mM/L)	Glucose utilized mg/0.04 ml cells/ hour	Lactate utilized mg/0.04 ml cells/ hour
Glucose	6	0.96 (2)*	0.27 (2)
Lactate	14		0.13 (2)

* The figures in parentheses are the numbers of experiments performed. The values obtained are averages.

Although the aerobic glycolytic metabolism of these cells is tremendous, approximately 25 times greater than that of red blood cells, the oxygen consumption still remains a sizeable portion of their total metabolism. These cells, with lactate as the substrate, have the high Q_{O_2} of 10–12.

The most fascinating feature of these tumor cells is the inhibition of oxidative metabolism when glucose is added in amounts above 1.5 mM (5). It is likely that a similar concentration of glucose is maintained in the ascites fluid and cells of the peritoneal cavity of the mouse so that a maximum total amount of energy can be obtained by the cells from glycolysis and oxidation. On the other hand, a shunting of the metabolism from oxidative to glycolytic may be an important factor in the abnormal growth and nondifferentiation of cells. The elucidation of this phenomenon may be helpful in characterizing so-called "autonomous" growth.

In this regard it is interesting that, although added lactate increases the oxygen consumption, it has a slower rate of disappearance (Table 4) than lactate formed metabolically from glucose. Perhaps this indicates a greater utilization of 3-carbon materials for protoplasmic synthesis when glucose is the substrate. The possibility of an inhibition of lactate metabolism by the D-isomer is being investigated. Experiments with C^{14}-glucose and lactate are planned to confirm and extend these findings.

SUMMARY

With the use of the Ehrlich mouse ascites carcinoma cell (a variant of the Ehrlich breast carcinoma), a washed tumor cell system was obtained which could be employed for chemical and biological studies.

1. The oxidative metabolism of this system was not appreciably influenced by wide changes in total ionic strength or by variations in ionic pattern. The metabolism was, however, adversely affected by a lowering of the pH below 7.4, and irreversible enzyme damage was apparent above pH 7.4.

2. The quantity of cells in the system could be determined by direct count, by packed cell volume measurement, or by dry weight determination, the latter two being better criteria for quantitation.

3. The washed tumor cell system had a lowered concentration of exogenous substrates and, after 1 hour of respiration, showed a lowered oxygen consumption. The "endogenous" respiration could be markedly stimulated by adding glutamate, lactate, pyruvate, oxalacetate, malate, succinate, α-ketoglutarate, citrate, aspartate, or alanine.

4. The addition of small amounts of glucose to the cell system stimulated, whereas large quantities inhibited, oxidation. Glucose was utilized at a rate of about 0.96 mg/hour/0.04 ml (17×10^6) tumor cells.

5. Lactate was utilized at a rate of about 0.13–0.27 mg/hour/0.04 ml cells. The higher rate was found when glucose was the substrate—this in spite of the diminished oxidative metabolism.

ACKNOWLEDGMENTS

The authors wish to thank Miss Ellen L. Marston for her technical assistance in the study.

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Announcements

Applications for Grants in Cancer Research

The Committee on Growth of the National Research Council, acting for the American Cancer Society, is accepting applications for grants-in-aid in support of cancer research. Applications for new grants received before October 1 will be considered during the winter, and grants recommended at that time will become effective July 1, 1954. Investigators now receiving support will be notified individually regarding application for renewal of these grants.

The Committee feels that a clear understanding of cancer must rest upon a deeper insight into the nature of the growth process, normal and malignant. Therefore, the scope of the research program is very broad and includes, in addition to clinical investigations on cancer, fundamental studies in the fields of cellular physiology, morphogenesis, genetics, virology, biochemistry, metabolism, nutrition, cytochemistry, physics, radiobiology, chemotherapy, endocrinology, and environmental cancer.

During the past year the American Cancer Society, on recommendation of the Committee on Growth, has awarded approximately 250 grants totaling more than $1,700,000. A program of similar magnitude is contemplated for the coming year.

Application blanks and additional information may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, Washington 25, D.C.

Establishment of "Transplantation Bulletin"

Within recent months, efforts have been under way to increase the exchange of information in the field of tissue transplantation among investigators in laboratories and those at the bedside. The USPHS sponsored a small conference which took place in October, 1952, at Arden House, Harriman, New York. At this conference workers in plastic surgery, cancer, zoology, and other fields discussed common problems in tissue transplantation. In March, 1953, the Ciba Foundation sponsored a similar conference in London, England, which emphasized the "Preservation of Normal Tissues for Transplantation." Participants at both conferences felt that some means should be provided for further continuous exchange of information on an informal basis among investigators in the clinic and the laboratory interested in problems of tissue transplantation.

To meet this need, it is proposed to issue a quarterly "Transplantation Bulletin." This Bulletin will serve several functions.

1. It will maintain, and publish, at least once yearly, a Transplantation Registry which will list all practicing physicians and research workers, both in the United States and abroad, interested in transplantation problems. The Registry will cover the fields of plastic surgery, endocrinology, cancer, genetics, immunology, experimental morphology, etc.

2. The Bulletin will provide a medium for a rapid and informal exchange of information on problems and progress in the clinics and laboratories both here and abroad. It is emphasized that the Bulletin will not publish formal papers. However, brief comments on unreported data will be welcomed.

3. It will, through a staff of Corresponding Editors, attempt to keep all members of the "Transplantation Registry" informed of forthcoming meetings of the professional societies, at which there will be presentations dealing with any aspects of transplantation. It will also try, through its Corresponding Editors, to have the subjects covered in a coordinated fashion at the meetings.

4. It will maintain a bibliography in the fields listed in item (1) above.

The Transplantation Bulletin hereby extends an invitation to all workers in the fields of medicine and biology interested in tissue transplantation, to submit their name and field of interest to E. J. Eichwald, M.D. University of Utah College of Medicine, Salt Lake City, Utah. A subscription fee of $3.00, to cover the expenses of printing the Bulletin and maintenance of the Registry and bibliography, will be payable after the first issue of the Bulletin has appeared. The first issue is expected to appear in August, 1953.

The Editorial Board of the Bulletin

Gordon Research Conference on Cancer

August 31–September 4

1953

FINAL PROGRAM

Monday, August 31:

A. Morning Conference

1. The Effect of Glucosamine on Tumor Growth.
   J. H. Quastel and A. Cantero, Montreal General Hospital and Notre Dame Hospital, Montreal.
2. Studies of Oxidative Metabolism of Tumors *in Vivo*.
   Harris Busch, Yale University School of Medicine, New Haven, Conn.

B. Evening Conference

1. Character of I^{131}-Induced Pituitary Growth; Hormonal and Histological Changes Accompanying Acquisition of Autonomy.
   Jacob Furth, Oak Ridge National Laboratory, Oak Ridge, Tenn.
2. The Progression of Experimental Tumors.
   L. Foulds, Royal Cancer Hospital, Buckinghamshire, England.

Tuesday, September 1:

A. Morning Conference

1. Lipid Metabolism in Tumor-Bearing Animals.
   Frances L. Haven, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.
2. Lipogenesis and Lipoprotein in Tumors.
   Robert Olson, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pa.

B. Evening Conference

1. An Antitumor Preparation from a Soil Microorganism.
   a) Laboratory Investigations. *C. Chester Stock, Donald A. Clarke, John Ehrlich and Quentin Bartz, Sloan-Kettering Institute for Cancer Research, New York, N.Y. and Parke-Davis Company, Detroit, Mich.*
   b) Clinical Trials. *David A. Karnofsky, Memorial and James Ewing Hospitals, New York, N.Y.*

Wednesday, September 2:

A. Morning Conference

1. Cytochemical Studies during Carcinogenesis.
   Bjorne Pearson, University of Vermont Medical School, Burlington, Vt.
2. Submicro Enzyme Chemistry of Normal and Neoplastic Cells.
   M. J. Kopac, New York University, New York, N.Y.

B. Evening Conference

1. The Biologic Activity of Desoxyribose Nucleic Acids as Hereditary Determinants in Bacteria.
   Hattie E. Alexander, College of Physicians and Surgeons, Columbia University, New York.
2. Recent Studies on the Chemistry of Heredity Determination in Bacteria.
   Stephen Zamenhof, College of Physicians and Surgeons, Columbia University, New York, N.Y.
3. Some Recent Observations on the Mammary Tumor Inducing Agent.
   Leon Dmochowski, College of Physicians and Surgeons, Columbia University, New York, N.Y.

Thursday, September 3:

A. Morning Conference

1. Epithelio-mesenchymal Interaction in Cultured Embryonic Rudiments of the Mouse.
   Clifford Grobstein, National Cancer Institute, Bethesda, Md.
2. Transplantation of Embryonic Nuclei and the Problem of Nuclear Differentiation.
   Robert Briggs and Thomas King, Institute for Cancer Research, Philadelphia, Pa.
3. Business Meeting.

B. Evening Conference

1. The Physiology of Normal and Leukemic Leukocytes.
   Edwin E. Osgood, University of Oregon Medical School, Portland, Ore.
2. Attempts at Sequential Antagonism in Experimental Neoplasia.
   Howard Skipper, Southern Research Institute, Birmingham, Ala.

Friday, September 4:

A. Final Conference

1. Cancer Endemiology.
   Johannes Clemmesen, Danish Cancer Registry, Copenhagen, Denmark.
2. Clinical and Experimental Studies on the Etiology of Lung Cancer.
   Ernest L. Wynder, Sloan-Kettering Institute for Cancer Research, New York, N.Y.

Book Reviews

Man against Cancer. The Story of Cancer Research. By DR. I. BERENBLUM. Baltimore, Md.: The Johns Hopkins Press, 1952. Pp. 182.

According to the Preface, this book was written primarily to give the layman factual information on the nature, diagnosis, and treatment of cancer and to stimulate his interest in the progress of cancer research. For those laymen with sufficient background the book will fulfill these objectives well. However, many laymen may find that their scientific backgrounds are inadequate to the task of reading and comprehending the book, even though their interest may be great. This is unfortunate, but discussion of so complex a subject necessarily entails the use of some scientific terms and concepts. The understanding of the terminology is aided by a glossary at the end of the book. The book will be of greatest value to beginning medical students and graduate students in the medical sciences who wish to obtain a broad introduction to the problem prior to more specialized study.

The book is well written, and the generalizations are carefully drawn. The discussions on clinical cancer present a sane approach which is too often lacking in the material written for the general public. The chapters on the influence of heredity and the frequency of the human disease are particularly well developed. In the section on cancer research Dr. Berenblum has made a real effort to present the evidence both for and against various theories and ideas, although his opinion is easily discerned. The discussions on the mutational theory of cancer induction, the hope for finding accurate diagnostic tests for early cancers, and the likelihood of developing general chemotherapeutic agents are notable in this respect.

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Sovetskoe Meditsinskoe Referativnoe Obozrenie ("Review of Soviet Medical Literature") Onkologia ("Onkology"), Vol. 1, 1949 (pp. 92); Vol. 2, 1949 (pp. 58); Vol. 3, 1950 (pp. 100). V. I. KAZANSKY (Ed.) Medgiz, Moscow.

These three volumes present in abstract form the Soviet literature on cancer for the years 1947, 1948, and 1949. Some 900 papers and books are briefly reviewed, giving the authors, full titles, and original references, and short summary-conclusions of the articles. Each volume is divided into a section on General Oncology, which includes articles on etiology and pathogenesis of tumors and on general diagnosis and therapy of tumors, thus encompassing experimental laboratory work. Approximately one-fourth of the volumes are devoted to subjects in this wide field. The second section is on Special Oncology, and is subdivided according to types of neoplasms, as follows: (*a*) central nervous system, (*b*) peripheral nervous system, (*c*) eye, (*d*) upper respiratory passages, including ear, tongue and mouth, (*e*) lungs, heart, and mediastinum, (*f*) breast, (*g*) esophagus and gastric cardia, (*h*) stomach, liver and intestines, (*i*) female genital organs, (*j*) male genital organs, (*k*) large intestine, (*l*) urinary system, (*m*) bones and joints, (*n*) skin, (*o*) lymphatic system, (*p*) blood vessels, (*q*) endocrine organs, and (*r*) rare tumors. The papers abstracted in this section are primarily clinical in context. There is also a section on "Prophylaxis" and the "Combat against Malignant Neoplasms," or on organizational and public health matters in cancer. A small section on "History" is included in two of the volumes.

These volumes and subsequent ones of the series, should be of importance to all workers in the field of cancer, as they make available in convenient form extensive reference material which otherwise would be most difficult, if not impossible, to trace and to use.

It may be of interest to note that an article by Ruchovsky (Vrachebnoe delo, pp. 482, 1949) "establishes" that a Russian pathologist named Rudnev is the real father of pathology instead of Virchow, and that a Russian veterinarian named Novinsky in 1877 successfully transplanted tumors in dogs and in horses, thus antedating Hanau by at least a decade. This chauvinistic note is a reflection of a general Soviet policy, which has extended into oncology.

Incidentally, "Reviews of Soviet Medical Literature," starting with 1947, are also prepared in thirteen other medical fields, including normal and pathologic morphology and embryology, normal and pathologic physiology, internal diseases, surgery, obstetrics and gynecology, but not including radiology.

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